❐ Part I ❐

General Microbiology

Contents

Introduction to the Student

The step-by step procedure found in the activities of this Practical motes will allow you to use your laboratory time more efficiently and makes the course more self-instructional. The more self-reliant you become, the greater your interest in microbiology will be. This new approach to learning depends on your being honest in evaluating yourself, since your instructor will only infrequently measure your mastery of the material. Therefore, your honest self-evaluation of your mastery of each module, is vital to your success. Do not be satisfied with partial understanding. Nobody knows better than you do whether you are "getting it".

Discussion provides explanation of theories and procedures, describes specific aspects of each module's topic, explains each objective, and prepares you for performing the activities.

Figures are used throughout the text to give visual explanations of complex techniques, schemes, and expected results. Illustrations are especially helpful in learning techniques. You must study them and imitate them exactly. *Tables* show you how to collect data and record them.

Worksheets include drawings, charts, thought questions, conclusions, and the like. Worksheets for each module requiring them are placed immediately after the module .

These components support and reinforce the objectives and make this presentation a self-taught course. This method gives you the choice of working at a slower or faster pace , without feeling pressured. Speed of performance is not a measure of solid learning.

Each module contains essentially single technique or concept that is explained by the discussion, activities, and post test. In modules that deal with difficult techniques, the activities may seem a first to be overdone, but they will save your time in the long run. For example, the aseptic tube transfer of bacteria is a technique that is difficult to learn, but it is the single most important technique to learn in microbiology since it protects you , your neighbor, and your pure cultures from contamination. If you do the practice activities thoroughly, you can learn such difficult-to-grasp techniques in one lab period.

As you perform the activities in the modules, you will be asked to place descriptions, drawings, some tables, and scientific conclusions on your worksheets. From this collection of data your instructor can then determine if you have made the correct observations and learned the procedures that the activities were designed to teach you. Your worksheets must be complete at the end of the semester for you to be successful in the course.

Periodically in the course, a MODS (module objective discussion session) may be scheduled. This is an informal discussion period, conducted by a student leader or the instructor, during which you explain the specific objectives to the other students and discuss them together. If you prepare for the MODS by reviewing your completed modules and worksheets, these specific objective discussions are a very useful learning aid.

Helpful Hints

1- The biggest mistake you can make with materials that you submit to your workbook is merely to copy the figures in each module. Many of these figures are included to help you determine whether you are seeing the correct organisms and/or structures through your microscope. It is to your advantage to make your drawings as you see them so that you will be able to recognize and identify them when you see them through the microscope again. In summary , don't dry-lab . It is very easy for your instructor to determine whether you are giving some thought and effort to your drawings and descriptions.
2- In modules without a related experience (designed to expand your learning) , try to invent one yourself. Discuss this related experience with your instructor before you proceed.
3- Materials and cultures for each lab session will be made available to you in a designated area. Less frequently used materials and equipment will be kept in specified storage areas.
4- Always read the label of the various dehydrated media. Learn the reason each medium is designed differently.

Laboratory Rules

Certain rules should be followed while you are working in the laboratory. Some are listed here. Your instructor may add other appropriate rules or suggest more safety procedures to remember.

1- Never eat or drink in the laboratory, and avoid putting objects in your mouth. Remember that you are working with living microorganisms, most of which are harmless, but others, if ingested , can cause you physical discomfort.
2- Disinfect your working area and wash your hands thoroughly at the beginning and end of each laboratory period. Wash your hand before you leave the lab for any reason, even a coffee break.
3- If you spill living microorganisms , cover the spilled material with paper towels, and pour your laboratory disinfectant over the towels and the contaminated area. Wait 15 minutes before you clean it up.
4- If your are injured (burned or cut), notify your instructor immediately.

A drawer, tray , cabinet, or other storage area will be assigned to you for the equipment that you will be using almost daily . You will also be assigned a microscope. It is your responsibility to keep it clean and to report any malfunctions to your equipment and microscope. This is most important because you may be sharing them with students in other lab sections . Suggested materials to supply for yourself:

1- Protective garment such as a lab coat .
2- Glass marking pencil.
3- lb coffee can for storage and incubation of culture tubes .
4- Hot pads or pot holders .
5- Colored pencils (red. blue, and green).

You are expected to read the assigned modules before attending each laboratory period. This will allow you to use your time efficiently, which is important

since you will be performing different stages of two or three different modules during the same lab period. That is , you may be preparing the media and reagents for one module, inoculating for another module, and collecting the results for a third module .

These self-instructional modules are presented in such a manner that you should be able to proceed with the activities (if you have preread the module) without a lengthy discussion and explanation from your instructor. Upon your arrival at the lab. you should be ready to go to work. Students who preread and preorganize their laboratory time will inevitably finish their labs early while learning more.

MODULE 1

Compound Microscope for the Study of Microbes

Discussion

Learning to use your microscope correctly is one of the most valuable accomplishments in your study of micr oes. In microbiology, the size of the organisms studied requires that you become an expert microscopist. You can accomplish this only with much practice or experience. This module is designed to show you how to use a microscope, not why a microscope magnifies to the extent it does or how the image is formed.

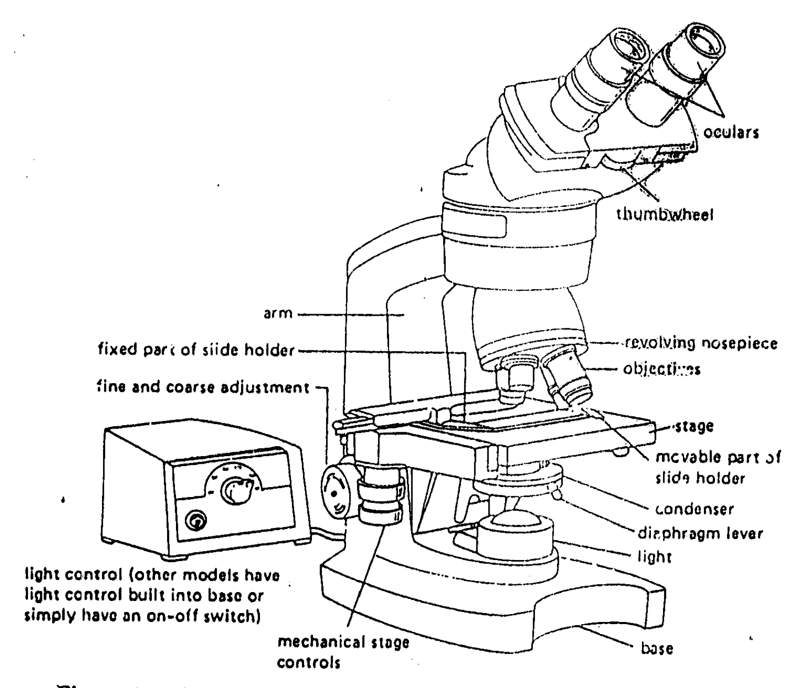


Figure 1-1 : Reichert-Jung (AO Spencer) series 150 binocular microscope (left front view)

The microscope illustrated in this module is a Reichert-Jung (AO Spencer) stirred. 150 binocular microscope. (The nosepiece moves to focus. In other brands the stage moves for focusing). Other makes of modern microscopes will be very similary. Study Figures 1-1 and 1-2 . You should be able to name all the labeled parts and explain their functions. (See Table 1-1) . It is important at this point to study the figures and table. (If your microscope is monocular all parts will be the same except the number of oculars and the thumbwheel.)

Each of the three objectives on your microscope has a number etched on it :10x, or 100x. This number indicates the number of times an object is magnified or enlarged by the objective. The 10x objective is the low-power objective, and it magnifies an object 10 times its actual size. The 45x objective (high power) magnifies an object 45 times its actual size, and the 100x objective (oil immersion) magnifies

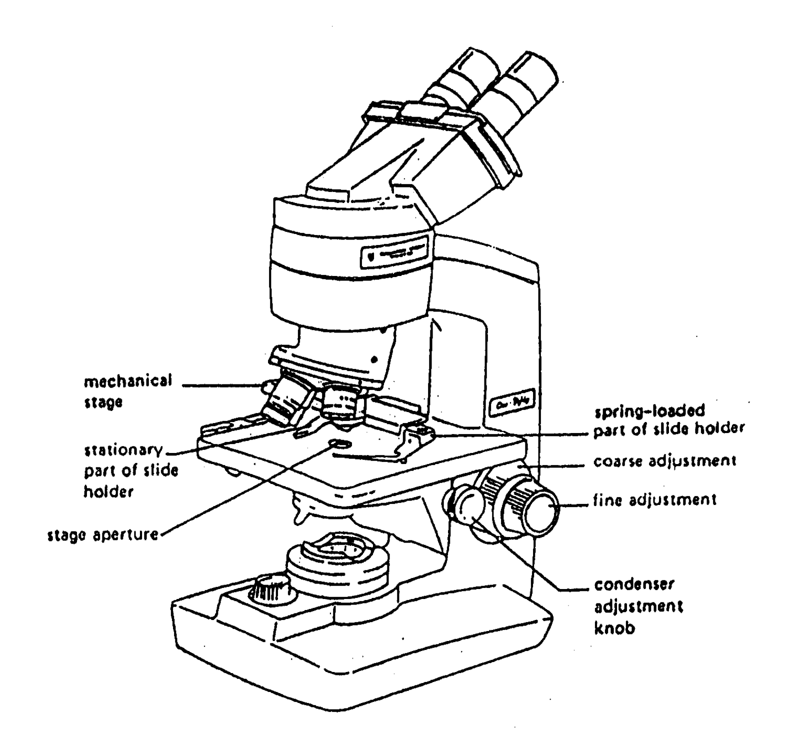


Figure 1-2 : Reichert-Jung (AO Spencer) series 150 binocular microscope (left front view)

Table: 1-1 : The Binocular Microscope .

Part	Function
Oculars (eyepieces)	Aseries of lenses that usually magnify 10
Thumbwheel (may not be present on other brands)	Can be rotated change from one objective to another and is raised and lowered in focusing .
Revolving nosepiece	Can be rotated to change from one objective to another and is raised and lowered in focusing.
Objectives	Usually three magnifications (if no scanning lens is present): 10x,low power; 45x, high dry power;and 100x, oil immersion. Powers of the objectives are distinguished by colored bands on the objectives.
Slide holder	Spring-loaded portion allows placing the microscope slide in the mechanical state, where it is held tightly.
Nosepiece	Is raised and lowered in focusing.
Diaphragm lever	Opens and closes the diaphragm to control the amount of light striking the object.
Condenser	Condenses light waves into a pencil-shaped cone, thereby preventing the escape of light waves. Also controls light intensity when raised and lowered.
Condenser adjustment knob	Raises and lowers condenser
Mechanical stage	Allows the slide to be moved.
Mechanical stage controls	Move the slide on two horizontal planes, that is, back and forth and side to side.
Base	Supports entire microscope.
Light intensity control	Turns light source on and off and controls light intensity.
Arm	Supports upper half of the microscope.
Coarse adjustment	Moves the nosepiece up and down rapidly for purposes of approximate focusing.
Fine adjustment	Moves the nosepiece up and down very slowly for purposes of definitive focusing.

100 times the actual size. Some microscopes are equipped with objectives that vary from the usual 10x, and 100x combination. The most common variation is a 10x, 44, and 97x combination. Some microscopes are equipped with a 3.5x scanning lens in addition to the other three objectives.

The objectives are often marked with incised bands around the lower part to allow you to distinguish one from another quickly and easily without finding the magnification number each time. This is especially important for the high-power and oil-immersion objectives, which are nearly the same length. The high-power objective (45x) is meant to be used dry, and the outer lens could be damaged by immersing it in oil accidentally. If the objectives are coded with bands, the 10x usually has one band, the 45x has two bands, and the oil-immersion objective (100x) has three bands. The

bands are sometimes colored for still easier recognition, in which case the oil-immersion objective is usually banded in red.

Your ocular or eyepiece also magnifies, usually 10 times. Thus the magnification at eyepoint (That is, what you see in the microscopic field while looking through the ocular) is magnified 10 times more than the magnification marked on the objective you are using.

Ocular	Objective	Magnification at eyepoint
10 times x	10 x =	100 x
10 times x	45 x (44x) =	450 x (440x)
10 times x	100x (97x) =	1000 x (970x)

The objectives on your microscope, and on most microscopes, are *parfocal*. That is , they are mounted so that when an object is in sharp focus with one objective, it will be in approximate focus with the other objectives when they are rotated into working position. Thus if you have an object in sharp focus on low power, you can rotate the high-power objective (45x) into working position and achieve sharp focus with only a slight turning of the fine adjustment knob.

Despite the fact that the objectives are parfocal , the novice microscopist frequently "loses" the object when switching to a higher-power objective. This happens because each increase in power of magnification *decreases* the microscopic field by about one-half. The illuminated field *appears to* be the same size at all powers, but the area actually being magnified shrinks each time you switch to an objective that magnifies to a higher power. So if the object is not in the center of the field when you switch to a higher power, you can easily lose it from the resulting diminished field. Your task can be made much simpler if you remember always to center the object before you switch to a higher power.

In general, the more the magnification is increased, the more light enter the optical system . This module contains a practice activity designed to help you master light control, which is critical to good microscopy. It is to your advantage and well worth your time to repeat the practice activity until you master light control with your microscope.

Care of your Microscope

Read and reread this carefully. The following instructions and precautions will be most important to you in regard to the rapidity with which you become a skilled microsocpist . It would be advisable to memorize them.

1- Always use both hands when you carry your microscope. Grasp the microscope arm firmly with one hand , and lift it carefully. Place your other hand under the base of the microscope for support as you carry it. Keep your microscope vertical; the oculars could fall out if the microscope is tilted.

2- Each time you use your microscope, clean the optical system (ocular lens objectives, and condenser lens) before and after use. This is especially necessary if you must share your microscope with another student in another lab section. Use *only* optical lens tissue to clean the optical lens tissue to clean the optical

system . To remove oil or dust from other portions of your microscope, use soft cloth or facial-type tissue. *Always keep your microscope immaculate.*

3- *Never* remove any parts of the microscope without consulting your instructor.

4- When you have finished using your microscope for the day and have cleaned it properly, if your microscope does not have an autofocus stop, put your low-power objective into working position because it is shorter than the other two objectives and therefore less likely to be damaged by striking the mechanical stage accidentally. Replace the dust cover before returning your microscope to the storage cabinet.

5- If your microscope does not have an autofocus stop, *never* focus downward while looking through the eyepiece. To prevent breaking slides and possibly damaging the objective, you should always turn the objective down to its lowest point while watching from the side before you look through the eyepiece and begin to focus. Do not touch the lenses of the eyepiece. Skin oils can mar the polished glass surface of the lens.

QUESTIONS

A. **True or False Statements:** Circle the correct response.

T F 1. If two objectives are parfocal, one will be in approximate focus when the other is in sharp focus.

T F 2. The size of the microscopic field remains constant regardless of the magnification of the objective you are using.

T F 3. Magnification at eyepoint (i.e., total magnification) equals the magnification of the objective multiplied by the magnification of the ocular.

T F 4. It is safe to use facial-type tissue to clean the optical parts of your microscope.

T F 5. Once you have achieved an image, you should move the optical parts that control the light striking the object as little as possible.

T F

T F 6. Yeast cells are smaller than bacterial cells.

B. **Multiple Choice:** Select the best answer for the following statements.

7 ___ . The most commonly used ocular is:

a. 5X
b. 10X
c. 12X
d. 15X

8 ___ . If the total magnification with a 45X high-dry objective is 225X, what would be the magnification of the ocular?

a. 5X
b. 10X
c. 12X
d. 15X

9 ___ The resolution of a microscope is increased by:

a. using a shorter wavelength of light
b. decreasing the amount of light emerging from the diaphragm
c. using a condenser with low numerical aperture
d. choices a and c

C. **Matching:** Match the parts of the microscope with their functions.

____ 11. Ocular
____ 12. Revolving nosepiece
____ 13. Objectives
____ 14. Diaphragm lever
____ 15. Condenser
____ 16. Mechanical stage
____ 17. Base
____ 18. Arm
____ 19. Coarse adjustment
____ 20. Fine adjustment
____ 21. Body tube

a. Opens and closes diaphragm to control amount of light striking object
b. A series of lenses that usually magnify 10 times
c. Condenses light waves into a cone, thereby preventing escape of light waves; also controls amount of light striking object
d. Raised and lowered in focusing some microscopes
e. Usually has 3 magnifications
f. Supports upper portion of microscope
g. Rotates to change from one objective to another
h. Moves stage or body tube up and down rapidly for purposes of approximate focusing
i. Allows the slide to be moved
j. Supports entire microscope
k. Moves stage or body tube up and down very slowly for purposes of definitive focusing

D. **Completion:**

22.-30. Complete the following table with regard to *your* microscope.

Objective	Objective Magnification	Ocular Magnification	Total Magnification
Low			
High-dry			
Oil immersion			

31.-33. List the 3 morphological types of bacteria.

31. ____________________
32. ____________________
33. ____________________

Module 2

Simple Stain

Discussion

The chemical compounds used to stain bacteria are called dyes. We stain bacteria to make them more readily visible, since unstained cells are practically transparent. Therefore, for microscopic observations, stained bacteria are most often used.

Dyes can be acidic or basic. Acidic dyes such as acid fuchsin and eosin have a strong affinity for basic portions of the cell, that is, for the cytoplasmic components of the cell that are more alkaline in nature. Basic dyes such as crystal violet, methylene blue, and safranin have a strong affinity for the acid portions of the cell. The surface of a bacterial cell has an overall acidic characteristic because of a larger number of carboxyl groups located at the cell surface. The carboxyl groups (COOH) are the acid portions of amino acids, and many amino acids are combined in the cell wall. Therefore, when ionization of the carboxyl groups takes place, the surface of the cell has negative charges. For example,

$$-COOH \xrightarrow{\text{Ionization}} COO^- + H^+$$

In nature, however, the hydrogen ion is replaced by another positive ion such as Na^+ or K^+ and the H^+ binds with oxygen to form water. The surface of a bacterial cell could be represented as in Figure 2.1

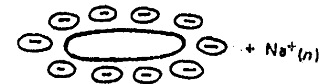


Figure 2-1 : The predominance of negative charges on the cell surface.

Basic dyes are commercially prepared as salts. For example, when we purchase methylene blue, it is actually methylene blue chloride. When rehydrated, the methylene blue chloride ionizes to have a positive charge on the colored part of the molecule, that is , MB+. It is this action that allows us to say that methylene blue is basic because in electrolysis, MB+ will move to the negative electrode. It is a law of chemistry that unlike charges attract each other, so the MB+ molecules are ielectronically bonded to the negative charges at the surface of the bacterium. When this happens, the cell is stained. A word equation to show the staining process can be written as follows:

Bacterial cell surface $-Na^+ + MB^+Cl^- \rightarrow$ Bacterial cell surface MB+NaCl

The staining of bacteria with a simple stain is , therefore, an exchange of positive and negative charges between molecules to form an ionic bond. If only a single dye is used to stain bacteria, we call this a simple stain. In a later module, you will be doing a differential stain that employs two dyes of different colors.

Activity

Caution: Many dyes are potential carcinogens. All stains should be handled carefully to avoid spilling them on your skin. It is advisable to handle stain covered slides with slide forceps and/or to wear surgical gloves when working with them.

Staining Bacterial Smears with a Simple Stain

1- Place Slide 1, on your staining rack.
2- Flood the slide with methylene blue. Allow the methylene blue to react with the smear for 1 minute.

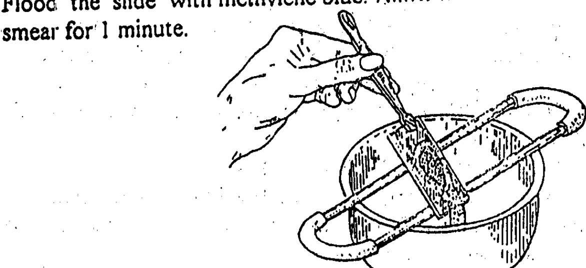


Figure 2-2 : Draining a bacterial smear before rinsing.

3- Using your slide forceps, tilt the slide so that the stain runs off into the staining pan or staining sink as shown in Figure 2-2.
4- Wash all the excess stain off while the slide is still held tilted over the staining receptacle tap.
5- Blot the smear gently with bibulous paper to remove the water.
   - Do not rub. Rubbing will remove the stain.

Repeat this staining procedure with the remaining five smears. If you stain more than one smear at a time , do not allow the stain to dry on the smear because the drying stain will crystallize and obscure the bacteria. You can avoid crystallization of the stain by using an excess of stain to flood the slide and by rinsing the stain off immediately with water , that is while the stain is draining off the smear.

After you have stained all six smears and allowed them to dry, examine the stains under the oil-immersion objective. *Use the oil-immersion objective as described before* . Attempt to determine the correct quantity of cells needed to make a "good" smear. Remember for future stains the number of loops from broth cultures and the amount of growth from a slant culture that yielded a "good" smear in your judgment. You will be making smears throughout the rest of this course, so this is important to you.

Formula for Reagent

0.5% Methylene Blue

methylene blue chloride	0.5 g
distilled water	100.0 ml

Dissolve the methylene blue in the distilled water.

Module 3

Gram Stain

Discussion

Microbiologists find the Gram stain a most useful aid in the identification of bacteria. The Gram stain is a differential stain requiring a primary stain and a counter stain stain

The primary stain is crystal violet, which is followed by an iodine solution. The iodine is called the *mordant* (a specialized term used in dyeing), which is substance, often a metallic compound , that combines with a dye to form an insoluble colored compound. This insoluble precipitate is called the crystal violet-iodine complex in the Gram stain procedure., Gram-positive organisms do not retain the primary dye-(after depolarization) if the iodine mordant is omitted. After depolarizing usually with 95% ethanol or acetone alcohol, a safranin counter stain is applied to the smear. If the acetone alcohol decolorizer step is omitted from the Gram stain procedure, all bacterial will appear Gram-positive.

Organisms that resist decolorizing and retain the crystal violet-iodine complex *appear purple or deep blue* microscopically and are called *Gram-positive* (G+)

Conversely, cells that decolorize or give up the crystal violet-iodine complex more rapidly will accept the safranin counterstain and *appear red* . These are the Gram-negative (G-) organism.

In 1884 , Christian Gram accidentally discovered what eventually was called the Gram staining reaction. He was working on the etiology of respiratory diseases at the Municipal Hospital in Berlin. As he attempted to stain biopsy specimens to differentiate microorganisms from the surrounding tissues, he applied crystal violet and then Lugol's iodine as a mordant. Both these solutions were standard reagents used in laboratories at that time . The precipitate formed was so thick that Gram used 95% ethanol as a clearing agent . He found that the tissue cells decolorized much more rapidly than the bacteria present in them. Gram originally thought he had developed a differential stain for all bacteria in tissue, but he soon observed that some bacteria did not retain the primary stain but were decolorized with the tissue cells and accepted the counterstain. Hence there was no differentiation between these bacterial cells and tissue cells. This presents no problem, however, when bacterial cells are stained by themselves; that is , when they are not in tissue cells.

Most living cells, including animal tissues, are Gram-negative. It is the Gram-positive characteristic that is distinctive. Some bacteria, yeasts and a few molds possess the Gram-positive characteristic.

Today there is still no single, universally accepted explanation for the differences in Gram reactions of certain cells. There are many theories to explain the differences, but none of them is completely satisfactory. The theory now considered most valid relates the Gram reaction to a difference of permeability of the cell wall based on structural differences. Gram-negative cells have a greater lipid content in their cell walls than Gram-positive cells. Lipids are soluble in alcohol and acetone, which are used a s decolorizers in the Gram stain procedure. The removal of the lipid by the decolorizer is

thought to increase the pore size of the cell wall, and this would account for the more rapid decolorization of Gram-negative cells.

Another theory suggests that a crystal violet-iodine-ribonucleate complex may form in Gram-positive cells but not in Gram-negative cells. This theory implies that the ribonucleic acids of the Gram-positive cell cytoplasm must be different and therefore bind more firmly with the crystal violet-iodine complex. The chemical bond formed with the G+ ribonucleate is not readily broken by the decolorizer.

You should remember that the differentiation of the Gram reaction is not an absolute, all-or-none phenomenon. It is based on the *rate* at which the cells release the crystal violet-iodine complex to the decolorizer. Even Gram-positive organisms can show a Gram-negative reaction if decolorized too much. A number of other factors can result in variable Gram reaction, such as the following.

1- Improper heat fixing of the smear. If a smear is heated too much, the cell walls can rupture, causing G+ cells to release the primary stain and accept the counterstain. This supports the theory that the Gram reaction is dependent on cell wall structure.
2- Cell density of the smear. An extremely thick smear may not decolorize as rapidly as one of ordinary density.
3- Concentration and freshness of the Gram staining reagents.
4- Length and thoroughness of washing after crystal violet, and the amount of water remaining on the smear when iodine is added.
5- Nature, concentration, and amount of decolorizer applied.
6- Age of bacterial culture . Gram reaction are reliable only for cultures up to 24 hours sold. This too, is related to cell wall integrity and permeability .

It is important to keep these variables as possible to ensure reliable and consistent Gram differentiation's. For this same reason, you should practice your Gram staining procedure repeatedly until you are confident that you are able to obtain consistent reactions. The time spent now in practice will serve you well since you will be using this differential stain constantly in your study of microbes.

The Gram stain is an indispensable aid to the identification of unknown bacteria. This simple procedure will allow you to place any bacterium into one of five broad areas and , at the same time, eliminate the remaining five areas. That is , the organism will be either a Gram-positive rod, a Gram-negative rod, a Gram-positive coccus, a Gram-non reactive (including some G- spirilla). Gram-nonreactives are microorganisms that do not stain or stain poorly. The genus *Mycobacterium* and various spirochetes fall into this group. Acid-fast organisms are only weekly Gram-positive and are better studied by other staining procedures. You can see that your search has been narrowed rapidly by eliminating several large groups of bacteria through the use of this most helpful staining technique.

Gram-variables have also been recognized. Gram-variables are bacteria that may appear both Gram-positive and Gram-negative on the same smear. Some organisms lose their Gram-positively in older cultures, possibly causing this Gram-variability. Gram reaction given in this manual, and in most reference works, are for 24 hour cultures.

Diagnosis and treatment of bacterial diseases are facilitated by determining the Gram reaction of the causative organism. The Gram-positive bacteria include the causative organisms of anthrax, rheumatic fever, diphtheria, botulism, septic sore

throat, and boils. A few representatives of the Gram-negative group are the organisms **causing** chlorea, typhoid, dysentery, whooping cough, certain types of food poisoning, **bubonic** plague, and many other diseases.

In later modules, you will be involved in activities that demonstrate **the correlation** of the Gram reaction with the following important physiological **and cytologic** traits: -

1- G^+organisms are generally more susceptible to penicillin, disinfectants, and dye bacteriostasis (inhibition of bacterial growth) than are G- organisms.
2- G- organisms are more sensitive to lysis and digestion by strong alkali, acids, and lysozyme (a cell-lysing enzyme).
3- G+ organisms tend to be more fastidious; that is, they have complex nutritional requirements for growth .
4- G+ organisms usually produce exotoxins, whereas most G- bacteria form endotoxins.
5- Gram-positivity is a characteristic easily lost, whereas Gram-negativity is never lost . Therefore, you will find Gram-negative cells in Gram-positive stained slides, but you should never find Gram-positive cells on a Gram-negative slide from a pure culture.

The first or second time that you do a Gram stain, you will probably find it easier to stain one slide at a time. After you are more familiar with the procedure, you will be able to put several slides on your staining rack and stain a whole series of slides together.

Activities

Activity 1 : Performing the Gram Stain

1- Prepare separate smears of *Bacilus subtilis, Escherichia coli, Staphylococcus aureus*, and *Neisseria Sicca* using the respective stock cultures.
   - You may put two smears on a single slide if you wish.
   - Remember to air-dry and heat-flix your smears, and label slides carefully.

2- Place labeled slides with heat-fixed smears on your staining rack over your stainless steel pan or other receptacle.
   - *Reminder:* Once you begin the staining procedure, you must *never* let a smear become dry until you have completed the procedure. By flooding the slide with an excess amount of stain each time you will prevent drying and consequent precipitation of crystallized dye on the slide, which can obscure the bacterial cells.

     Caution: Remember stains are potentially hazardous chemicals. Handle slides with forceps and/or wear surgical gloves while staining smears.

3- Perform the Gram stain as follows. See Figure 3-1 for a pictorial representation of this procedure. You may be using different staining bottles; the procedure, however is the same.
4- Flood the slide with *crystal violet,* and allow it to react for *1 minute.*
5- Handling the slide with your slide forceps, tilt it to about a 45° angle to *drain* the dye off the slide into the pan or staining sink as shown in Figure.
6- Continue to hold the slide at a 45° angle, and immediately rinse it thoroughly with a gentle stream of water from your wash bottle.

7- Replace the slide on the staining rack, and flood it with *iodine*. Allow the iodine to react for *1 minute*.

8- With your side forceps, tilt the slide, and allow it to *drain*.
9- Immediately *rinse* the slide thoroughly with water from your wash bottle
10- With your slide still held at a 45^{o} angle, *decolorize* it *quickly* by allowing the acetone alcohol to run over and off the smear. *Do not decolorize it too much*.
11- *Rinse immediately* with water from your wash bottle. This will stop the decolorizing process.
12- Replace the slide on your staining rack, and flood it with *safranin* counterstain. Allow the counterstain to react for *30 to 60 seconds*.
13- *Drain* the slide.
14- *Rinse* the slide throughly with water from your wash bottle.
15- Carefully *blot* your stained slide in your booklet of bibulous paper. *Do not rub*, as you could rub off a very thin smear.

Repeat this procedure until all four of your smears are stained.
Examine each smear microscopically. On the worksheet for this module, draw several representative cells from each smear as they appear under oil immersion. Label the drawings with the Gram reaction, cell shape, and arrangement.

If you feel you need more practice to perform the Gram stain procedure with consistency, ease, and confidence, you should repeat this activity as time permits.

Activity 2: Gram Stain Unknown Organisms

1- Prepare a smear of an unknown bacterium; air-dry and heat-fix it. Label the slide with the number of the unknown organism.
2- Prepare smears of the other unknown organisms in the same fashion.
3- Perform a complete Gram stain on these unknown smears, following the steps in Activity 1 (Figure 3-1).
4- Examine one of the stained smears microscopically, and sketch several representative cells as seen under oil immersion. List the Gram reaction, cell shape, and arrangement, if any.
5- Repeat step 4 with all unknown smears.

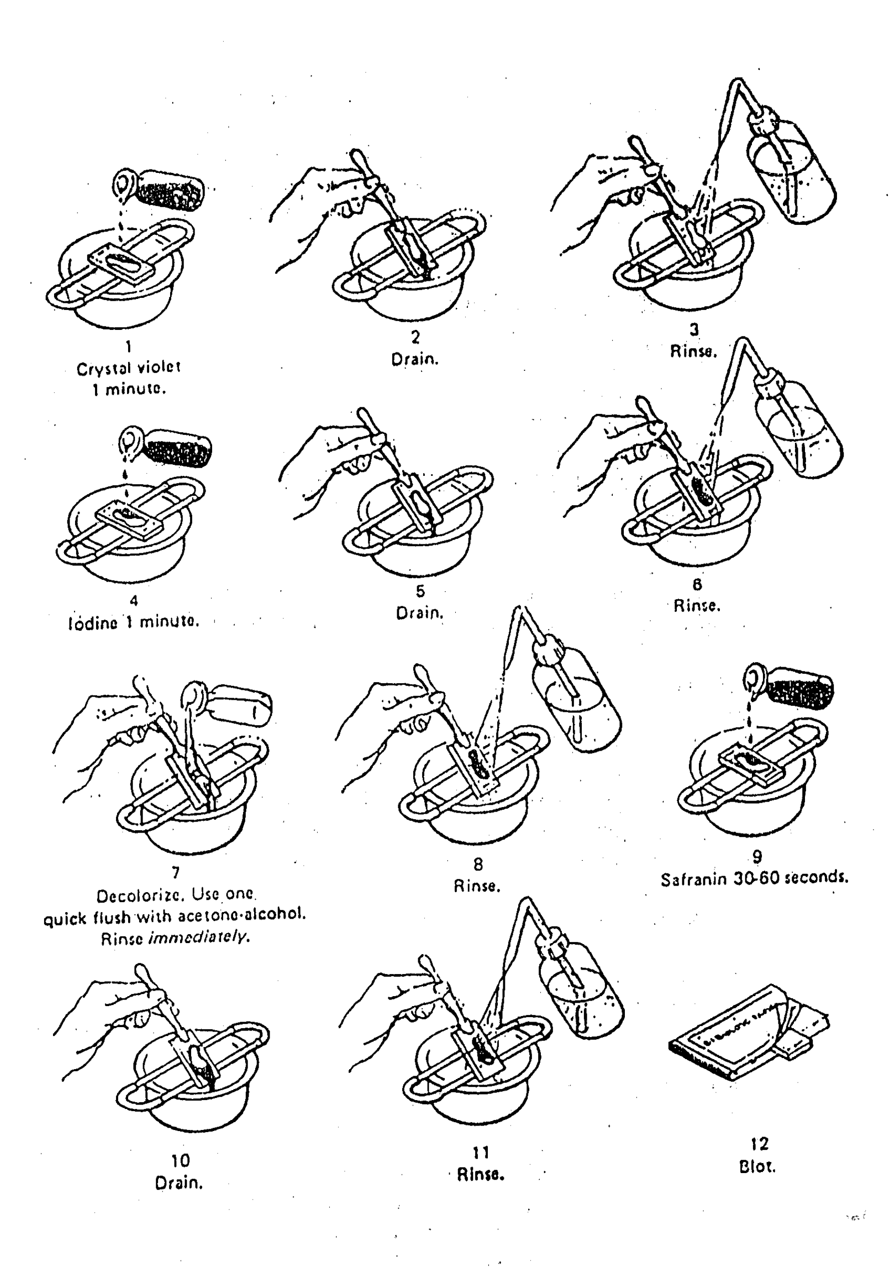


Figure 3-1 : The Gram stain procedure

- Many genera and species have the same Gram reaction and cell shape. Therefore, you *cannot* name unknown organisms from the Gram reaction only.

Cleck the numbers of your unknowns against your instructor's master list of unknowns to determine the accuracy of your Gram reaction and microscopic observations. If your Gram stain reactions and descriptions were not accurate, repeat this activity.

FORMULAE FOR REAGENTS

1- GRAM'S CRYSTAL VIOLET

Gram's crystal violet consists of two solutions.

Solution A

Crystal violet	2.0 g
ethanol, 95%	20.0 ml

Dissolve the crystal violet in the ethanol

Solution B

ammonium oxalate, C.P	0.8 g
distilled water	80.0 ml

Dissolve the ammonium oxalate in the distilled water

Make up solutions A and B separately Then pour the two solutions together, and mix well.

2- GRAM'S IODINE

Iodine, C.P.	1.0 gm
Potassium iodide, C.P	1.2 g
Distilled water	300.0 ml

Grind the iodine and potassium iodide together in a mortar until finely divided. Add water in small quantities to wash the contents out of the mortar. Add the rest of the water, and mix thoroughly. This solution should be stored in a tightly closed amber bottle.

3- GRAM'S SAFRANIN

Safranin O	0.25 g
Ethanol. 95%	10.0 ml
Distilled water	100.0

Dissolve the safranin in the ethanol. Mix throughly. Add the distilled water, and mix well. Filter the solution through filter paper.

4- ACETONE ALCOHOL

Ethanol, 95%	70.0 ml
Acetone	30.0 ml

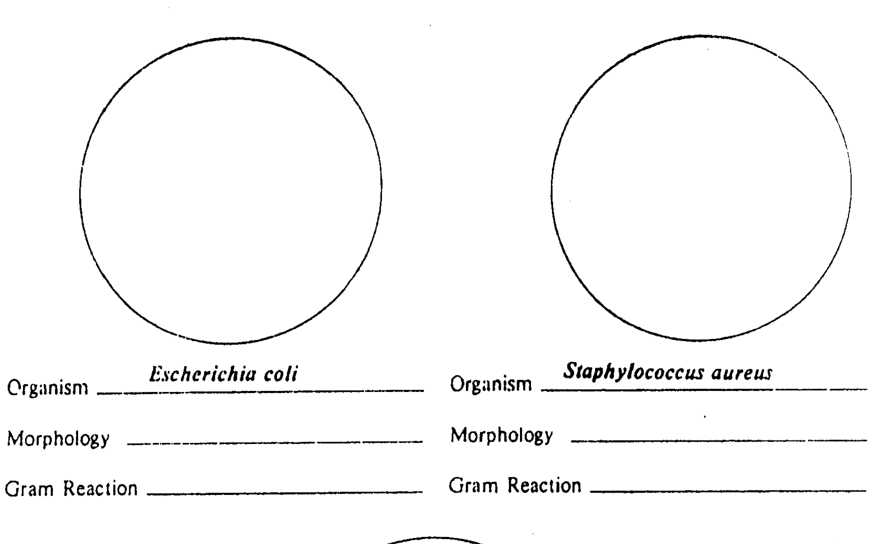
Mix the two liquids thoroughly.

Dispense these four Gram stain separate dropper bottles, labeling the dropper bottles appropriately. Store the remainder of each reagent in large tightly stoppered or screw-cap bottle. As your working bottles (dropper bottles) become empty, replenish the reagents from the large storage bottles.

Name ______________________ Date ________ Grade ________

The Gram Stain

·RESULTS AND OBSERVATIONS



Organism *Escherichia coli*

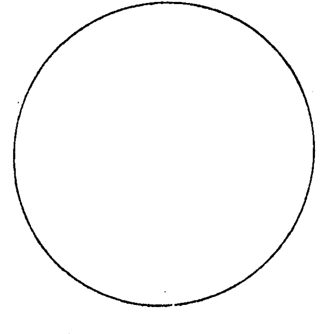
Morphology ______________________

Gram Reaction ______________________

Organism *Staphylococcus aureus*

Morphology ______________________

Gram Reaction ______________________



Organism *Bacillus cereus*

Morphology ______________________

Gram Reaction ______________________

)UESTIONS

.. **True or False Statements:** Circle the correct response.

T F 1. Over decolorization in the Gram staining method results in all the cells being Gram-positive.

T F 2. The cell wall of Gram-positive organisms appears to be less permeable to the effects of the decolorizer because it has a lower lipid content than the cell wall of Gram-negative organisms.

T F 3. Gram-negative and Gram-positive cells look the same under the microscope after decolorization of the first two reagents of the Gram stain.

T F 4. Sporeforming bacteria are, for the most part, Gram-negative.

T F 5. The Gram reaction of a specific bacterial species will never vary if the staining procedure is consistent.

T F 6. Gram reactions are reliable only for cultures 24 hours old or younger.

T F 7. Excessive washing can remove the primary dye or dye-iodine complexes within the cells.

T F 8. All smears are heat fixed unless contraindicated by the procedure.

T F 9. The amount of inoculum is the most important factor in making a "good" smear.

B. Multiple Choice: Select the best answer for the following statements.

____ 10. Which of the following organism pairs are Gram-negative?

a. *Staphylococcus aureus* and *Mycobacterium phlei*
b. *Staphylococcus aureus* and *Pseudomonas aeruginosa*
c. *Escherichia coli* and *Serratia marcescens*
d. *Micrococcus luteus* and *Corynebacterium xerosis*

____ 11. The application of which reagent must be timed most carefully in the Gram stain?

a. acid-alcohol
b. iodine
c. crystal violet
d. acetone-alcohol
e. safranin

____ 12. The organisms in a Gram-variable smear can be:

a. only Gram-positive
b. only Gram-negative
c. either Gram-positive or Gram-negative
d. initially Gram-negative and then Gram-positive
e. none of the above

____ 13. Which of the following can result in a Gram-variable reaction?

a. too thick of a smear
b. over decolorization
c. age of cultures
d. inconsistent application of technique
e. all of the above

C. **Completion:**

14. A preparation made by mixing a loopful of water and a bit of an agar slant culture on a glass slide is called a ______________________.

15.-16. Gram-negative bacteria stain ______________________ and Gram-positive bacteria stain ______________________ in a completed Gram stain.

17. The Gram stain is a ______________________ (type) stain.

18. All members of the genus *Neisseria* are Gram ______________________.

19. Endospores appear ______________________ in a completed Gram stain.

20.-35. Fill in the table below to show the changes in Gram-positive and Gram-negative bacteria during each major step of the staining process.

Reagent	**Purpose**	**G+ Organisms**	**G- Organisms**

MODULE 4

Capsule Stain

Discussion

Most bacterial cells secrete a viscous substance that accumulates around the outside of the cell and "coats" the cell wall. This structure, depending on the thickness of the layer and its viscosity, and its demonstrability , is called the *capsule* or the *slime layer*. Most bacteria secrete at least some slime that is more soluble and less viscous than a capsule. The capsule appears as a larger definite structure and can therefore be more easily demonstrated.

The size of the capsule is influenced by the environment in which the organism is cultured. For example, tryptose phosphate agar induces the production of larger capsules than does nutrient agar . It is also true that capsule producers, *in vivo,* produce large capsules if they are disease-causing bacteria.

The bacterial capsule has significance both for the bacteria and for humans. The capsule serves a protective function for the bacterium by acting as an osmotic barrier between the cell body and the environment. The capsule also appears to interfere with the phagocytic action of leukocytes when encapsulated bacteria invade the human body. There is speculation that the bacterial capsule may also be a reservoir of stored food or for disposal of waste products.

For some disease-producing organisms, the virulence and infectiveity of the organism are increased by , or entirely dependent on , the presence of the capsule. For example , *Streptococcus pneumonia* becomes a virulent when it loses the ability to produce capsules.

The bacterial capsule i s usually composed of polysaccharides that are water-soluble and nonionic. As learned, most staining techniques are based on chemical bonding between ionized particles of the dye , molecules and ionized areas on the surface of the cell. That is , an ionic bond is formed by the attraction of unlike charges on the dye molecules and the cell surface, and the bacterium is stained. Because the bacterial capsule is nonionic, it connote be stained in the usual manner.

Since we cannot stain the capsules, techniques have been developed that allow us to stain the background and leave the capsule clear. This is called a *negative stain.* In essence, a negative stain dyes everything *except* the structure you wish to visualize.

The phase microscope actually provides the best method for visualizing bacterial capsules. However , phase equipment is expensive and not always readily available. A similar effect can be achieved by preparing a wet mount of the bacterium and adding carbon particles (India ink) to the suspension. When this preparation is examined microscopically with reduced light intensity, a phase-like effect is achieved. The capsules appear to be halos or clear rings around the bacterial cell, and the background looks dark, as shown in Figure 4-1. This method can be completed very rapidly but is a temporary preparation. The difficulty with a wet mount i s that you must observe it with your high dry objective, which magnifies only 450 times.

A variation of this technique is the modified Gin's stain for capsules. This method allows you to use your oil-immersion objective and magnify 1000 times. It differs from the wet mount demonstration of capsules in that it employs a negative

stain and a positive stain. When examined microscopically, the background is dark, and the capsules appear as clear, unstained rings (negative stain), with the small purple cell body in the center of the rings (positive stain), as shown in Figure 4-2 .

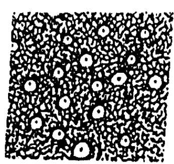


Figure 4-1 : Phase contrast preparation of *Klebsiella pneumoniae*

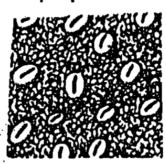


Figure 4-2 : Gin's method capsule stain of *Klebsiella pneumoniae.*

This variation is not restricted by the shortcomings of the wet mount preparation. The counter stain makes the cell body more clearly visible and allows you to compare the size of the cell to that of he capsule.

Either of these capsule stain techniques can be readily adapted for use with a broth culture by simply omitting the water when you prepare the suspension of organisms and carbon particles. Be sure that the organisms are well dispersed in the broth .

ACTIVITY

Modified Gin's Capsule Stain Using the Blood Smear Method

1- Mix India ink, *Klebsiella pneumoniae,* and water on the end of a clean slide, as shown in Figure 4-3a.
   - Use your inoculating loop to add the bacteria to equal amounts of India ink and water.
2- Place the edge of a second slide in the mixture. Allow the mixture to run across the base of the second slide, as in Figure 4-3b.
3- While holding the second slide at an acute angle, push the mixture toward the opposite end of slide 1, as in Figure 4-3c.
4- allow to air-dry (do not heat-fix).
5- Using your staining equipment, flood the smear with crystal violet and let it react for 1 minute.
6- Drain and rinse the smear.
   - Some of the smear may wash off as you rinse it. Do not be dismayed. Remember that this smear is not heat-fixed.
7- Put one end of the slide on a paper towel, and prop the slide up at a 45^{o} angle.

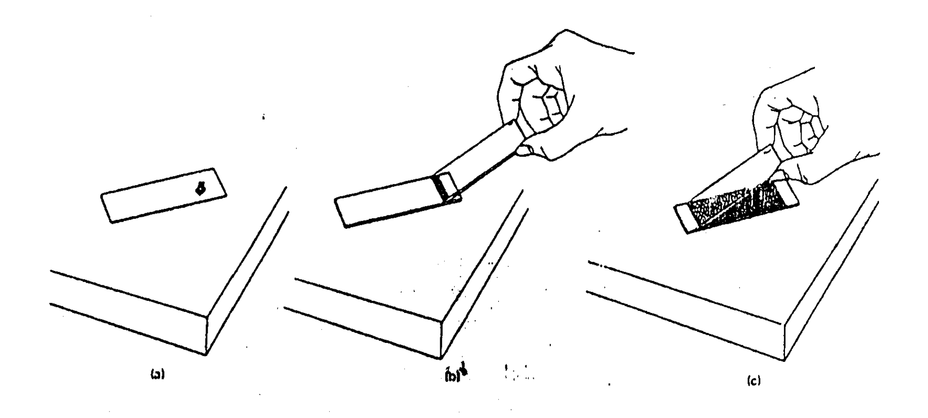


Figure 4-3 : Modified Gin's capsule stain procedure.

When making a capsule stain from a broth culture, it is not necessary to use a drop of water. Simply mix two parts of broth culture to one part of India ink and proceed as usual. The broth preparation should be mixed very gently to avoid disrupting any characteristic cell grouping, such chains and so on.

Examine your modified Gin's method slid under oil immersion, and draw representative cells on the worksheet for his module. Label the organism, the cell body, and the capsule. Then take the post test.

MODULE 5

Acid-Fast Stain

Discussion

FORMULAE FOR REAGENTS

1- ZIEHL-NEELSEN'S CARBOLFUCHSIN

basic fuchsin	0.3 g
ethanol, 95%	10.0 ml
phenol crystals, C.P	5.0 g
distilled water	95.0 ml

Dissolve basic fuchsin in the ethanol. In a separate container, dissolve phenol crystals in water. Mix the two solutions together thoroughly.

2- ACID-ALCOHOL DECOLORIZER

hydrochloric acid, concentrated (37%)	70.0 ml
ethanol, 95%	97.0 ml

add the acid to the ethanol, and mix well.

3- LOEFELER'S METHYLENE BLUE

methylene blue chloride	0.3 g
ethanol, 95%	30.0 ml
distilled water	100.0 ml

Dissolve methylene blue in the ethanol. Add the distilled water, and mix well. Filter the solution through filter paper and funnel.

DISCUSSION

Paul Ehrlich developed the acid-fast stain in 1882 in his work with the etiological agent of tuberculosis, *Mycobacterium tuberculosis*. The Ziehl-Neelsen acid-fast stain procedure commonly used today is the result of changes in methodology to improve the original Ehrlich technique. The Ziehl-Neelsen reagents, in which carbolfuchsin is the primary dye and Loeefler's methylene blue is the counterstain, are much more stable than those of Ehrlich . The decolorizer is 3% hydrochloric acid (HCl) in 95% ethanol. This acid alcohol is a very intensive decolorizer and should not be confused with the weaker acetone alcohol used in the Gram stain procedure.

Most genera are not acid-fast , with the exception of the genus *Mycobacterium* and some species in the genus *Nocardia*. Both of these genera contain species that are pathogenic. The two best-known acid-fact human pathogens are *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and *Mycobacterium leprae* , the etiological agent of Hansen's disease or leprosy.

Human tuberculosis is diagnosed by clinical symptoms, X-rays, and laboratory findings. The specimens of choice for bacteriology studies of pulmonary tuberculosis are sputum and bronchial secretions. The tubercle bcillus must be demonstrated in the specimen by acid-fast stain and culture and by X-ray examination of the patient before the diagnosis of TB is definitive.

Generally, acid-factness is very rare in cells. Organisms of the genus *Mycobacterium* have a high fat content, containing relatively large amounts of lipid, materials including fatty acid waxes, and complex lipids. They have wax-like cell walls that are relatively impermeable. The same relative impermeability that is associated with acid-fastness gives the genus *Mycobacterium* an above-average resistance to disinfectants. These organisms are also quite resistant to drying and can survive for long periods in dried supmtum or other body fluids. These characteristics necessitate special precautions when caring of TB patients. However, TB bacilli are readily destroyed by pasteurization and by ordinary sterilization by heat.

Because of the waxy, impermeable cell wall, special measures are necessary to allow the primary stain to penetrate. The primary dye (carbolfuchsin) is formulated with an aqueous 5% phenol (carbolid acid) solution as a chemical intensifier to assist penetration. Heat is also applied to the stain-covered bacterial smear as a penetrating agent. As in the Schaeffer-Fulton spore stain, the primary stain is driven into the cell by steaming.

Once the stain has penetrated the cell wall, the acid-fast cell retains it through very intensive depolarization. Therefore, acid-fastness means that once a cell is stained with carbolfuchsin, it resists decolorization with an acid decolorizer. Other genera of bacteria would lose the primary stain immediately upon exposure to an acid decolorizer. Decolorizing in the acid-fast stain is *not* the delicate procedure that it is in the Gram stain. Acid-fast cells retain the primary stain and appear red microscopically, whereas the non-acid-fast organisms will accept the counterstain and appear blue.

Activity

Coution: Remember to treat all stains as potentially hazardous chemicals.

Ziehl-Neelsen Acid-Fast Stain

1- Especially make a *mixed* smear of *Mycobacterium smegmatis* and *Staphylococcus epidermidis.*
   - Take a small amount of organisms from the *M.smegmatis* slant and a small amount from the *S.epidermidis* slant and mix both organisms together in a drop of water on a slide. Emulsify the suspension as well as possible.

2- Allow the smear to air-dry, and heat-fix as usual.

3- Flood the slide with carbolfuchsin stain.

4- Heat the stain-flooded slide to steaming by inverting your Bunsen burner and passing the flame over the pooled stain several times, as shown in Figure 5-1. When you see steam rising from the stain, remove the burner. When the steam stops rising, pass the flame over the stain again periodically as necessary to keep the stained smear just at steaming. *Do not boil or allow the smear to dry.* As stain evaporates form the slide, replenish with additional carbolfuchsin. *Steam for 5 minutes.*

5- Allow the slide to *cool* to prevent breaking the slide when you rinse it with cool water.

6- Tilt the slide to drain it, and rinse it thoroughly with water.

7- Flood the slide with acid alcohol and allow it to decolorize for 15 to 30 seconds. Then tilt the slide to a 45^{o} angle, and add decolorizer drop by drop. If red color

continues to come off in the decolorizer, repeat the flooding with acid alcohol. When the red color no longer comes off in the decolorizer, perform the next step in this procedure. It is difficult to over decolorize mycobacteria.

8- Rinse the slide with water.

9- Replace the slide on your rack, and flood it with Loeffler's methylene blue counterstain. Allow the stain to react for 1 minute.

10- Drain and rinse the slide.

11- Blot the slide carefully in your pad of bibulous paper, or allow the slide to air-dry in the tilted position.

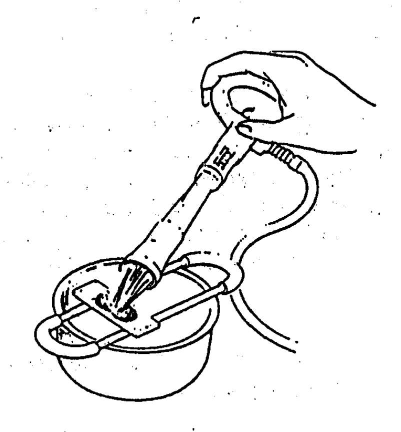


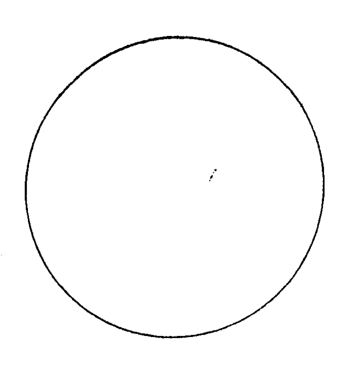
Figure 5-1: Heating the carbolfuchsin in the acid-fast stain.

Now examine your stained smear under the oil-immersion objective, and on the worksheet for this module, draw several representative acid-fast cells among the non-acid-fast *S.epidermidis*. Acid-fast bacilli are usually found in clusters and are long thin rods. Label your sketch appropriately. Remember that the acid-fast cells will retain the primary stain and appear red, and the non-acid-fast cells will appear blue because they have lost the primary stain and have accepted the counterstain.

Name ______________________ Date__________ Grade__________

The Acid-Fast Stain

RESULTS AND OBSERVATIONS



Staphylococcus aureus
+
Organisms *Mycobacterium smegmatis*

Magnification ______________________

Stain ______________________

QUESTIONS

A. True or False Statements: Circle the correct response.

T F 1. Acid-fast cells are quite common.

T F 2. Acid-fast organisms are relatively resistant to drying and to disinfectants.

T F 3. The Gram stain decolorizing agent is stronger than the one used in the Ziehl-Neelsen acid-fast procedure.

T F 4. Methylene blue serves as the primary stain in the acid-fast stain.

T F 5. The inoculating loop must be resterilized between cultures when preparing a mixed smear.

B. Completion:

6. Carbolfuchsin is prepared by adding ______________________ to basic fuchsin.

7.-9. The acid-fast stain identifies microorganism with a high ____________ content. The primary stain is not washed out by the ____________ decolorizer and the cells are said to be ____________.

10. Members of the genus ____________ are usually acid-fast.

11.-14. Name 2 pathogenic acid-fast bacteria and the disease that each one causes.

Organism	**Disease**
11. ____________	13. ____________
12. ____________	14. ____________

15.-26. Fill in the table below to show the changes in acid-fast and non-acid-fast bacteria during each major step of the Ziehl-Neelsen acid-fast method.

Reagent	Purpose	Acid-Fast	Non-Acid-Fast

B. Completion:

9.-20. Fill in the table below to show the changes in sporeforming and non-sporeforming bacteria during each major step of the Schaeffer-Fulton method.

Reagent	Purpose	Spores	Vegetative Cells

21. The Schaeffer-Fulton method is a ________________ (type) stain.

22.-24. The genus ________________ consists of anaerobic sporeforming bacteria. Some cause diseases such as ________________ and ________________.

25.-26. The genus ________________ consists of aerobic sporeforming bacteria. Some can cause the disease ________________.

27. Sporeforming bacteria are ________________ (morphology).

28.-30. Identify the location of the endospore in each of the following drawings.

Location

28. ________________

29. ________________

30. ________________

MODULE 6

Ubiquity of Microorganisms

Discussion

Although this module is designed to show that microbes are ubiquitous, it also introduces the necessity of using aseptic technique when you are working with bacteria in the laboratory. In Module 7 you will learn the skill of aseptically transferring bacteria from one tube to another. It is in this next module that you will actually manipulate living bacteria correctly, without fear of self-contamination or contamination of a pure culture.

After you complete this module, you will realize that the omnipresence of microorganisms is intimately associated with the use of aseptic technique while transferring bacteria. This will become even more apparent to you after you grow many different kinds of microorganisms from the different sources of your environment. You will believe beyond a doubt that microbes are indeed ubiquitous or omnipresent, which means that *they are eerywhere* ! They are at the bottom of the ocean, on ice-capped mountain tops, in hot sulfur springs, in milk, in drinking water, in every possible place on this planet.

You should become truly bacteria-conscious when you realize that you are surrounded by bacteria, fungi, protozoans, and other microorganisms. The activities in this module will prove to you that tiny, invisible, ever-reproducing, living microbial cells are all around you, on you, and inside you. This should prepare you for the next module as you will appreciate the real need for aseptic technique when you work in the microbiology lab.

Now gather together the materials you will need to perform the following activities *Precaution :* You will be working with sterile petri dishes containing two different agar media. Notice that they look alike. *Do not* remove your plates from the supply area until you have labeled them on the bottom so that you can distinguish between the two media. Petri dishes are always labeled on the bottom instead of the lid so that there will be on errors caused by interchanging the lids.

Activities :

Activity 1 : Growing Microorganisms from Environmental Sources

A. Growing Microorganisms from Soil

1- Label a tube of nutrient broth with your name, date, and activity number.
2- Step outside your lab and obtain one pinch of moist soil.
3- Drop the soil in the sub of sterile nutrient broth.
4- Put the soil-broth tube in a coffee can or culture tube basket.
5- Incubate at 30°C for 48 hours or at room temperature for a longer period of time.
6- After incubation, make a simple stain, observe with your microscope.

Make a drawing and written description on the worksheet for this module.

B. Growing Microorganisms from the Air

Microbial cells are so small and so light that they are constantly being wafted around you by air currents, or they can be hitching a ride on dust particles. To show this, do the following:

1. Label a sterile nutrient agar plate carefully as you did your nutrient broth tube.
   - Be sure to label the bottom of the petri dish.
2. Open your nutrient agar plate for 20 minutes.
   - When you expose this microbial nutrient, the airborne bacterial and fungal cells will settle on it and develop into colonies after incubation. Spout your nutrient agar plate where air currents are maximal . Your lab worktable is also a good place to find bacteria. You decide where you would like to open your plate.
3. After 20 minutes , close your agar pate and put it in the 30°C incubator upside down for 48 hours.
   - *Always* incubate culture plates in an inverted position unless otherwise instructed.

In your next lab session, draw several different colony types. Not all colonies will be bacteria; light, fluffy, cotton-like colonies are fungi.

C. Growing Microorganisms Obtained from Your Tabletop

After you have grown the bacteria obtained form your tabletop, you will understand why you should wipe down your working area with disinfectant at the beginning and at the end of each laboratory period. From now until the end of the course, make a habit of disinfecting your worktable before you begin your lab work and again after you have finished.

1- label the bottom of a nutrient agar plate carefully with your name , date activity number, and type of medium

2- Remove one saline-soaked swab from the tube, and rub the cotton tip on your tabletop before it is disinfected.
   - Try to include such places as areas around gas jets and comers where dust particles are present.

3- Use the contaminated swab to inoculate your nutrient agar plate.
   - It does not matter how you inoculate the nutrient; simply rub the cotton swab gently over the surface of the agar. Use as much of the surface of the nutrient as you can for the inoculation.

4- Return the cover to your plate and incubate inverted in the 30°C incubator for 48 hours.

5- Repeat this activity after you have disinfected your tabletop.

In your next lab session, draw the growth on the plates. The second plate should show a reduced amount of growth if disinfection has been effective.

D. Growing Bacteria *on* You

You can grow bacteria from any surface of your body at any time. To demonstrate this:

1- place your fingers lightly on the surface of the sterile nutrient agar in a petri dish, and drag your dingers gently back and forth across the plate two or three times.

2- Close the plate and incubate it, upside-down, at 30°C for 48 hours.

3- Wash your hands with soap and rinse well, but do not dry them.
4- Repeat steps 1 and 2 with a second plate.

In your next lab session, make drawings of the growth of these skin bacteria. This will require two drawings, one before and one after washing your hands.

E. Growing Bacteria *in* You.

Lab a tryptic soy agar (TSA) plate carefully and completely. TSA is preferred to NA as a nutrient medium for this activity because it is especially formulated to support the growth of streptococci and other organisms commonly present in your body that have more complex nutritive requirements (fastidious organisms).

All orifices of your body contain many different types of microbes. To demonstrate the bacteria in one of your body openings.

1- Touch your tongue to the sterile surface of a TSA plate.
   - The more surface area of your tongue that touches the agar, the more growth you can expect.
2- Close the plate and incubate it at 37ºC for 48 hours.
   - Note the higher temperature of incubation. This higher incubation temperature has a logical reason: microbes that flourish in you usually grow best at body temperature.

In your next lab session, make drawings of the colonies arising from your mouth microorganisms.

Activity 2: Continuation of growing Bacteria *on* You

1- Press your slightly open lips against the surface of a nutrient agar plate. Incubate for 48 hours at 30ºC.
2- If you bite your fingernails, try removing material from under your nails with a sterile nasopharyngeal swab.
   a. Moisten the swab by dipping it into the sterile saline you have left from Activity IC before you remove the material from under your nails.
   b. Again rub the swab over the surface of a sterile NA plate, rotating it as you rub. Incubate your plate.

After incubation, draw the growth seen on your plates.

Activity 3 : Your Conclusions on the Ubiquity of Microorganisms

After growing microbes from several different sources in your environment, summarize your bindings in short written form on your worksheet.

MODULE 7

Aseptic Transfer of Microbes

Discussion

Since microbes are omnipresent, which means they are present everywhere, it is necessary to use extreme care not to introduce unwanted organisms into a pure culture of infect yourself. Unwanted bacteria may introduced by direct contact with contaminated surfaces or your hands, that is, by actually touching the media or inner surfaces of the tube with any object that has not been sterilized. Since bacteria are also airborne, they can enter your tubes via air cruets. There are certain tested techniques that you can use to keep outside microorganisms from contaminating your transfer culture . These tested techniques are called aseptic techniques. These same techniques will also protect you from self-contamination and those around you from contamination in the laboratory. Laboratory accidents do not happen very frequently if you make it a habit always to practice good aseptic technique. If you learn to handle bacteria correctly, you will then have only the desired organisms growing in your transfer culture, and since you transferred a pure culture, you will get only this particular bacterium to grow . You will *not* have contaminated your pure culture. A pure culture is one in which only one single species is to be found.

This module shows, step by step, how to manipulate pure-culture transfers without adding outside, contaminating bacteria or infecting yourself and contaminating the lab. In transferring bacteria from one tube to another, you mount especially aware of airborne bacteria so that they do not contaminate the material you are working with.

Activities

Activity: 1: Practice of Aseptic Tube Transfer

When you master a certain tested technique, you will be able to transfer bacteria aseptically . You will be using these basic manipulations in most of your laboratory experiments throughout the laboratory portion of this course. Therefore, it will be to you advantage to master this technique *now*.

This technique requires much observation and practice. Carefully study Figures 7- through 7-15, which illustrate the approved technique. *Study the instructions accompanying the figures very carefully!*

Imitate the figures by doing several "dry runs," using the two empty culture tubes that are in your tube rack. Do one step at a time and check yourself. After you have imitated the entire series of figures, one step at a time, you will know what is meant by aseptic technique in tube transfer.

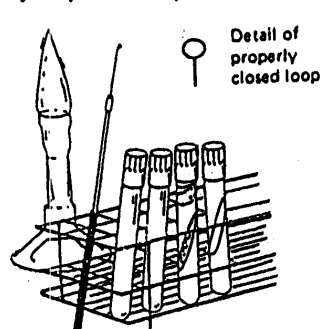


FIGURE 7-1
The necessary equipment. Be sure tho loop is closed (forms a circle).

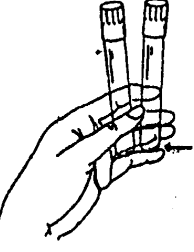


FIGURE 7-2
Hold both culture tubes in your left hand. (Hold them in your right hand if you are left-handed.) The tubes should *not* be held vertically once the closures are removed.

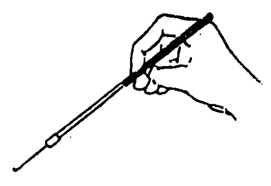


FIGURE 7-3
Hold your inoculating loop in your right hand. (Hold loop in your left hand if you are left-handed.) The loop should be held like a pencil.

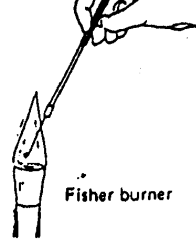


FIGURE 7-4
Flame the inoculating loop in the Bunsen (or Fisher) burner holding the loop upright so that all the nichrome wire gets red-hot at once. Allow the loop to cool so that you do not cremate the living bacterial cells you are about to transfer.

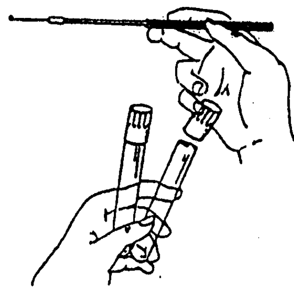


FIGURE 7-5
Remove the closures one at a time from both culture tubes. Do this by wrapping the little finger of your right hand around the closure of the tube nearest to your right hand. Grasp only the uppermost portion of the closure so that the open end does not touch the heel of your hand.

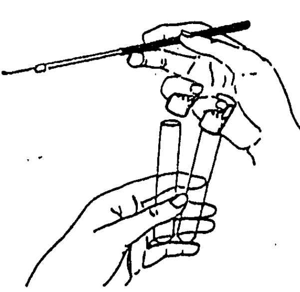


FIGURE 7-6
Now remove the second closure with the finger next to your little finger in the same manner, that is, by wrapping this finger around the second closure. Approach this tube by reaching between the two tubes. *Do not* attempt to reach behind the tubes.

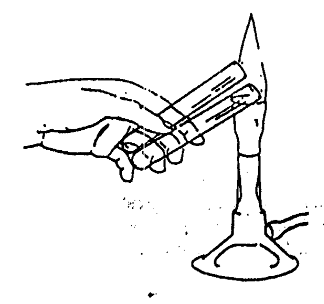


FIGURE 7-7
Flame the necks of the uncovered tubes by passing them back and forth through the flame twice. Be careful to hold the tubes in a nearly horizontal position.

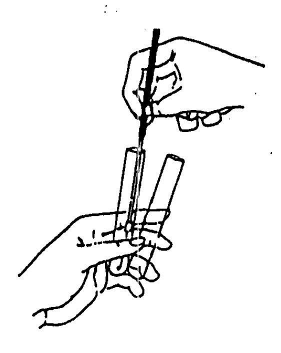


FIGURE 7-8
Insert the inoculating loop into the pure culture (or the practice tube substituting for it), and remove a small amount of bacteria. Note the position of the closures in the right hand.

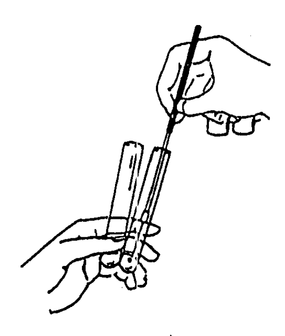


FIGURE 7-9
Transfer this inoculum (the small amount of bacteria) to the surface of the uninoculated slant (or the practice tube substituting for it).

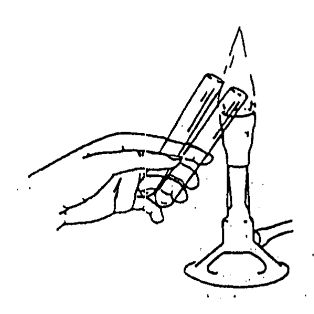


FIGURE 7-10
Reflame the necks of both tubes.

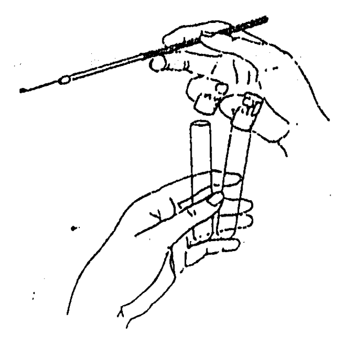


FIGURE 7-11
Recap the tubes, putting the closures on the same tubes from which they came. It is best to return the cap to the tube you uncapped first, that is, the closure held by your little finger.

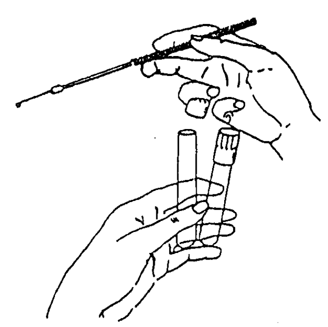


FIGURE 7-12
Then recap the second tube.

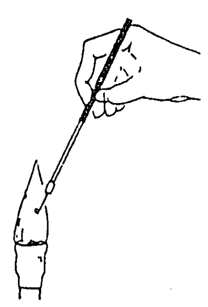


FIGURE 7-13
Reflame the loop, killing all the bacteria on it.

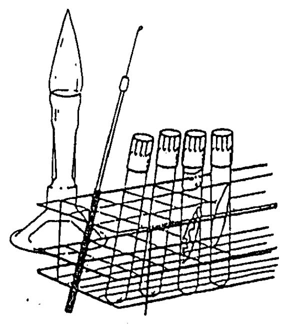


FIGURE 7-14
Return the tubes and the loop to the culture tube rack.

The checklist following the Figure summarize the manipulations represented by the series of figures. Once you have learned the correct handling of the equipment shown in the figures, you may need only to refer to the checklist for a reminder of the step-by-step procedure.

Checklist

1- Hold both tubes in your left hand and the inoculating loop in your right hand, as shown in Figures 7-2 and Figure 7-3 (Everything is reversed if you are left-handed).
2- Flame the inoculating loop until all the nichrome wire is hot. Allow the loop to cool for approximately 30 seconds to avoid cremating the bacteria you are about to transfer.
3- Remove the closures one at time from both culture tubes. Remove the closure closest to you right hand first by wrapping the little finger of your right hand around it. Remove the second closure with the finger next to the little finger on your right hand with the same wrapping motion. Approach the second tube by reaching between the two tubes. *Do not* attempt to reach behind the tubes.
4- Flame the neck of the uncovered tubes by passing the tubes back and forth through the flame twice. Be careful to hold the tubes at less than a 45^{o} angle while they are uncapped.
5- Insert the inoculating loop into the stock culture (or the empty practice tube substituting for it), and remove a *small* amount of bacteria.
6- Transfer this inoculum (that is , the *small* amount of bacteria) to the surface of the uninoculated slant (or the empty practice tube substituting for it).
7- Reflame the neck of both tubes by passing the tubes back and forth through the flame twice.
8- Recap the tubes, putting the closures on the same tubes from which they came. It is best to recap the tube nearest your right hand first.
9- Flame the inoculating loop.
10- Return the tubes and the loop to the culture tube rack.

Activity 2 : Demonstration for a Classmate (optional)

Using the same e empty culture tubes, have another student observe you as you demonstrate your aseptic technique. use the step-by-step procedure, referring to the figures and the instructions accompanying them as you proceed. Stop after each step so that your classmate can not on the checklist whether your technique for that step is correct. Repeat the instructions in the checklist until you can follow the procedure in a flowing succession of motions. This activity will help prepare you for the next.

Activity 3 : Dry run Demonstration for Laboratory Instructor

Practice enough dry runs so that the manipulations of aseptic tube transfer become a natural technique for you . Now demonstrate your aseptic technique for your laboratory instructor and have it approved before proceeding to Activity 4.

Activity 4 : Aseptic Transfer of Living Bacteria

You should now be ready to work with living microbes. As you know, *most* bacteria are nonpathogenic. However, the types that do cause disease can be very

harmful. Therefore, you need to protect yourself and your classmates by handling living bacteria correctly.

Each and every time you make an inoculation, you must label it carefully and completely. An adequate label should include your name, the date of inoculation, the name of the organism, the source of inoculum if appropriate (such as a throat swab), the type of medium, the temperature of incubation, and the module and/or activity number. See figure 7-16 for an example.

FIGURE 7-15
Three tubes and closures can be manipulated simultaneously once you've mastered this technique. One inoculum is sufficient to inoculate two or three tubes.

FIGURE 7-16
Proper label.

1- In your culture tube rack, place a slant of *Serratia marcescens*, a red pigment-producing bacterium and three uninoculated slants of nutrient agar.
2- Label your nutrient agar slant.
3- Using the technique you learned in your dry runs, transfer a small amount of living bacteria (inoculum) to the nutrient agar slants.
    - Take care not to cut or gouge the agar surface. Allow your loop to glide over the agar surface from the bottom of the tube to the top of the slanted surface with a slight side to side motion.
4- Incubate your slants at 30°C for 48 hours.

During your next lab period, examine your transfer slants for colony morphology and pigment production. If your aseptic technique was done correctly, the growth will appear smooth and confluent, and only red pigment producers will be present. Your aseptic technique has been successful if you have just one species of bacteria growing on your transfer slants, a pure culture of that species. Sketch the growth on your NA slants on the worksheet for this module.

Activity 5: Aseptic Transfer from a Slant Culture to Broth

1- Place a tube of sterile nutrient broth in your culture tube rack with the slant of red pigment-producing bacteria.
2- Label the tube completely, as in Activity 4.
3- Aseptically transfer a small amount of living bacteria to the tube of sterile broth.
- Immerse your loop, with the inoculum, in the broth, and shake it vigorously two or three times to deposit a few cells in the sterile broth.
4- Put the newly inoculated broth tube in a coffee can or basket in the 30°C incubator for 48 hours.

During your next lab period, examine the broth tube, and notice the turgidity and red pigment. Write your conclusions on aseptic transfer on the worksheet for this module.

MODULE 8

Streaking for Isolation

Discussion

Begommomg microbiology students find it difficult to streak for isolated colonies for the following two reasons:

1- They do not use as much of the streaking surface as possible. This results in fewer dilutions.

2- They use too large an inoculum, which means that their streaking must dilute thousands and thousands of cells until the individual cells are separated from one another.

The practice activities in this module are designed so that you will use *all* the streaking surface and make the maximum number of dilutions. This module also strives to help you think small enough so that you will not have too many bacterial cells in your original inoculum.

It is difficult to realize just how very minute bacterial cells are: each individual bacterium measures approximately 1/25,000 inch in size. Therefore, when you introduce an inoculating loop of bacteria onto the surface of a nutrient agar plate, you are actually placing tens of thousands of cells on the medium. A large inoculum contains so many bacterial cells that they cannot develop into individual colonies. Therefore, the growth is continuous over the entire surface. This is called confluent growth. When you learn to use a small amount of inoculum and learn how to separate these thousands of cell so that they are not touching one another, you have correctly streaked for isolation.

Proper streaking for isolation results in pure colonies. A pure colony arises from a single mother cell. After your nutrient agar plate is streaked, separating the bacterial cells from one another, these separated single cells are then called *mother cells.*

Upon incubation of your streak plate, each mother cell divides by asexual binary fission, that is, by a splitting in half of the cell, in 20 to 30 minutes, giving rise to two daughter cells.In the next 20 to 30 minutes, the daughter cells split i n half, and another generation of four new daughter cells comes into existence (see Figure 8-1).

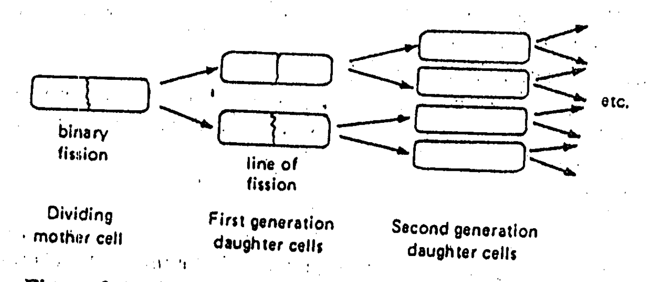


Figure 8-1 : Asexual reproduction (binary fission) of bacterial cells.

The new cells continue to divide in exponential numbers, resulting in billions of daughter cells. These billions of cells pile up on top of and around each other, and a pure colony is born. You must remember, however, that a colony can be considered a *pure* colony *only* if it does not touch another colony.

In actuality for most bacteria after 24 hours of incubation, a pure colony consists of 50 to 72 generations of cells arising from a single mother cell. A colony is therefore composed of billions and billions of daughter cells. Bacterial growth, then, mean an increase in cell numbers, not an increase in cell size.

In summary, after completion of this module, you will have developed the correct technique for separating bacterial cells from one another, thus allowing them to develop into pure colonies.

When you have completed the practice activities in this module, you will be able to streak for isolation. This is a basic microbiological technique that is important to your success in the laboratory portion of this course. Therefore, don not skip any of the practice activities designed to help you become adept at using this technique.

Activities :

Activity 1 : Simulation of Streaking for Isolation Using Paper and Pencil Using a pencil or pen, follow these steps, in which you will be imitating the procedure you will use when streaking for isolation on a nutrient agar plate with living bacteria. Keep in mind that when working with bacteria, you will be attempting to dilute the number of cells in each sector and that you will be *flaming* your inoculating loop between sectors. Flaming the inoculating loop kills the remaining cells on the loop and thereby aids in diluting the cells. Each sector represents a dilution or a reduction in numbers of the thousands of bacterial cells in the original inoculum. Sector 0 is for the original inoculum and represents thousands of cells. The steps of this procedure, 1 through 4, are always done in numerical order.

1- On plain sheet of paper, draw a circle about 3 inches in diameter.
2- Line and label the circle exactly as in Figure 8-2.
   - If you are left-handed, the 0 sector should be on the right: look for other reversals as you proceed through the steps of this technique.
   - Notice that the 0 sector is *smaller* in relation to I, II, and III. Sectors. I, II, and III should be almost equal in surface area.
3- Begin step 1 of the technique now (Figure 8-3). Using your pencil or pen, draw almost solid lines in Sector 0.
4- Study step 2 (Figure 8-4), and then draw lines from Sector 0 into sector I as shown.
   - Be careful not to let your lines in Sector I cross over each other or extend too near the boundary of Sector. I. As you begin drawing your looping lines. be certain that only the first two or three lines enter Sector 0. In doing this, you are imitating the dilution of the number of cells in Sector 0

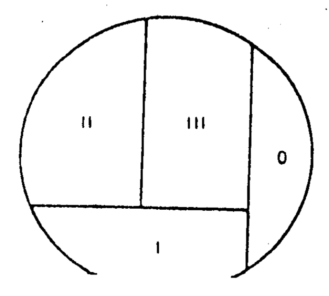


Figure 8-2 : Dilution sectors as drawn on paper.

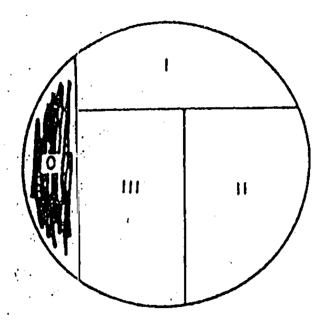


Figure 8-3

Step 1: Sector 0 represents the planting of a small amount of bacteria with your inoculating loop. This simulates the original inoculum.

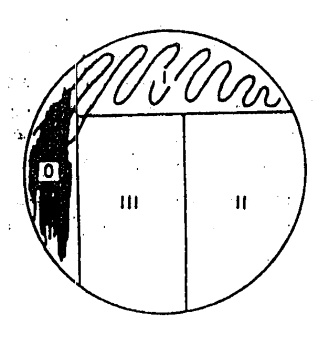


Figure 8-4

Step 2: Sector I represents the first dilution. Your lines in Sector I should be as uniformly apart as shown here.

5. Rotate your piece of paper counterclockwise one quarter turn so that Sector I is on the left.
6. Draw the streak lines as shown in Figure 8-5. This is step 3 of the procedure.
7. Rotate your piece of paper a quarter turn to the left again, and imitate the final dilution by streaking Sector III as shown in Figure 8-6, which is step 4.
8. Using another piece of paper, draw another circle similar to the one you have just finished. Mark and label it in the same manner. Repeat this practice activity several times. Proceed very carefully through all four steps. When you have done this to your satisfaction, show the final sketch to your lab instructor. If your lab instructor approves your work, you are ready to proceed to the next practice activity.

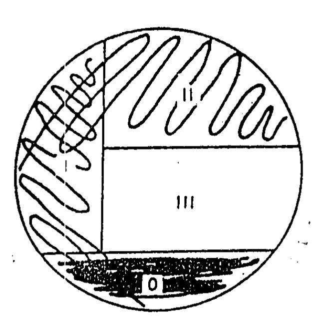


Figure 8-5:

Step 3: Sector II represents the second dilution.

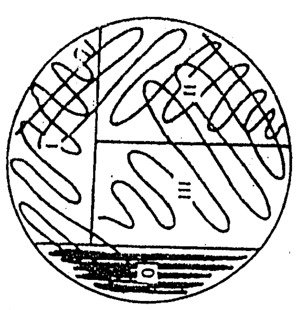


Figure 8-6: Step 4: Sector III represents the final dilution of the organisms. Be sour to leave some space between each sector to allow for the expanding bacterial growth

Activity 2: Dry Run Using Empty Petri Dish and Felt Pen

1- Gather together a petri dish, a felt pen , and a wax glass-marking pencil .
2- Turn the petri dish upside down, and mark i t *on the bottom* with the wax pencil as shown in Figure 8-7.
   - Take careful note of the different position of the sectors as compared with their position in Figure 8-2. *note well* that the position of Sector I is reversed while the petri dish is upside down; that is, Sector I is now on the bottom instead of on the top.
3- Next turn the unopened empty dish right side up.
   - The marking now appears as shown in Figure 8-8, and the sector positions are now exactly as in Figure 8-2.

Not that you have learned how to make the four-step dilution, in this practice activity pretend that the felt pen is your inoculating loop and that your empty petri dish contains a solid medium. When you remove the petri dish lid, you will want to protect your medium from airborne bacteria. Therefore, the petri dish lid should be removed just far enough to let you peek in and see where you are placing your felt pen and making your lines. Figure 8-9 shows you how to handle the lid with your left hand.

4- With the petri dish lying on the table in front of you , carefully remove the lid as shown in Figure 8-9.
5- With Sector 0 on the , left draw the streak lines on the inside of the glass bottom.
   - Refer to the four steps in Activity I, and follow them exactly
6- Proceed now through all four steps, reading the instructions as you go.
7- Close the petri dish, and have your streaking patterns checked by your laboratory instructor.

Activity 3: Streaking for Isolation Using Living Bacteria

1- Label and line the bottom of the plate containing agar just as you did in Activity 2
2- Next follow the steps shown in Activity 1: using your inoculating loop, transfer a *small* amount of bacteria from a slant culture of *Escherichia coli* to Sector 0 of you agar plate.
3- Proceed through you dilutions numerically (see Activity 1).
4- When you have finished, invert your plate, label it, and incubate it at 30°C for 48 hours.

Only after incubation will you know if you have successfully streaked for isolated colonies. If you have mastered the technique in this module, your streak plate should look similar to one of the plates in Figure 8-10. Isolated colonies often occur before Sector III. The sector in which isolation takes place is not important as long as you get separated colonies. Show your plate to your lab instructor for approval.

Precautions: The agar can be cut with the inoculating loop, so use light but definite touch. The loop should be kept as horizontal to the agar as possible. The inoculum need not be so large that you can see it macroscopically; simply touch your loop to the bacterial growth. *Flame your loop, and let it cool, between dilutions.* If colonies appear on any area of the agar surface that you did not streak, they are airbome contaminants and must not be used for subclturing. So examine the location of the colonies carefully.

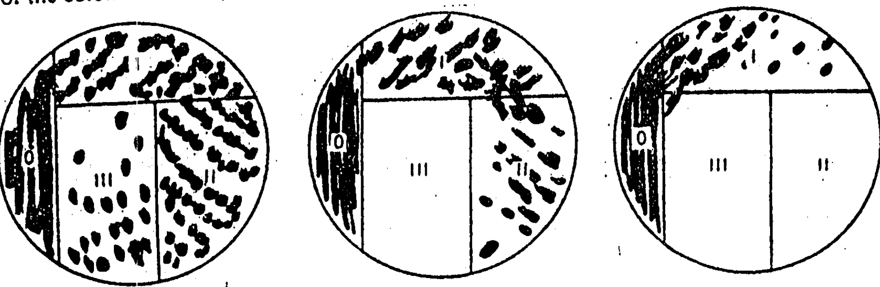


Figure 8-10: Incubated petri dish. After 48 hours of incubation, your plate should show isolated colonies. *Isolation may occur in any sector except Sector 0,* depending on the number of cells in the inoculum.

Activity 4: Use of a Cotton swab for Original Inoculum.

1- Begin this activity by dividing the bottom of the agar plate into the same sectors that you used in Activities 2 and 3.
2- Next hold both the *E.coli* broth tube and the tube and the tube containing the sterile swab in your left hand.
3- Using your best aseptic technique, remove the closures with your little finger and the one next to it.
4- Remove the sterile swab from the sterile tube with the thumb and forefinger of your right hand.
5- Flame the necks of the tubes.
6- Dip the swab into the broth culture tube, saturating it with *E.coli*
7- Flame the necks of the tubes again, recap them, and put them back in the tube rack.
8- You should still be holding the swab in your right hand, so carefully lift the lid of the agar plate and streak Sector 0 with the *E.coli* saturated swab.
9- Discard your swab in a container of disinfectant or in anther appropriate place.
10- Streak for isolation with your inoculating loop, using the same technique that you developed in the preceding activities.

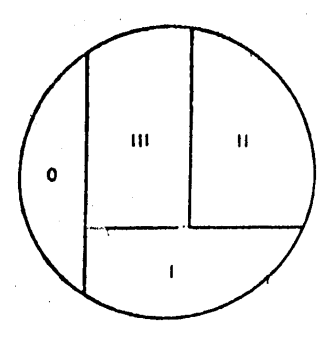


FIGURE 8-7
Dilution sector markings on the bottom of an empty petri dish.

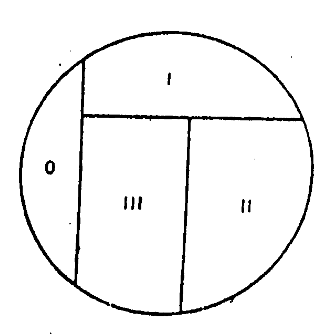


FIGURE 8-8
Dilution sector markings on the bottom of a plate as seen through the top of the empty petri dish. Notice that Sector I is again at the top when Sector 0 is at the left.

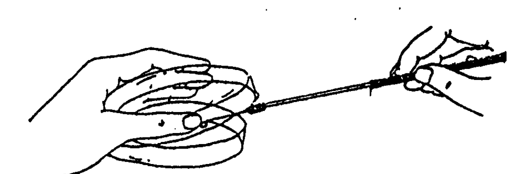


Figure 8-9: Proper handling of the lid of the petri dish. Open the petri dish slightly, but keep the lid over the dish. This will protect the sterile medium from airborne bacteria.

11- Incubate this plate with the plate from Activity 3.

At your next lab session, show al of your isolation plates to your lab instructor, and sketch them on the worksheet for this module.

SUMMARY OF STREAKING TECHNIQUES FOR ISOLATION

The following material can be used as a review in your future laboratory work. If you find that you do not get pure colonies when you streak for isolation, this summary should be of great help to you . Use it until you become so adept at getting pure colonies that it is no longer necessary for you to divide the plate into sectors.

When you can make three steak dilutions of the original inoculum that result in isolated colonies, discontinue marking the bottom of the plate into sectors. You have now mastered the skill of obtaining pure colonies and no longer need this instructional aid.

1- Mark the bottom of your nutrient agar plate with a wax pencil, dividing it into sectors as indicated in Figure 8-11.
   - If you are left-handed, the 0 sector will be on the right.
2- Next turn the marked plate right side up. The markings will be reversed and will look like those in Figure 8-12.
3- Carefully hold the lid in your left hand,* using it to protect the nutrient from airborne bacteria.
4- Using your inoculating loop and living bacteria, streak each sector in numerical order imitating the four steps shown in Figure 8-13.
   - Use a light but definite touch so that the loop does not dig into the agar but your streaks are uniform.
   - Be sure to flame and cool your inoculating loop before each dilution.

Always keep the sector form which you are streaking on the left.* of this by rotating the plate a quarter turn counterclockwise Note in Figure 8-13 that the arrow follows the correct position of Sector 0. Also note that Sector 0 (for the original inoculum) is small in comparison to the other sectors (I,II,and III) . This allows you to use the major portion of the plate for dilution of the numbers of bacteria in the original inoculum.

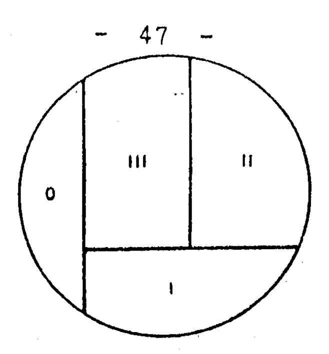


Figure 8-11: Sector markings on the bottom of an empty petri dish.

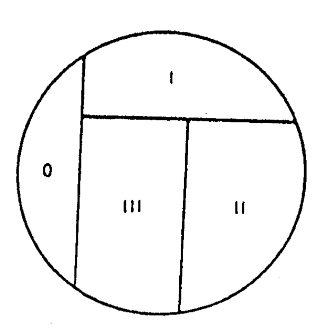


Figure 8-12: Sector markings on the same petri dish turned right side up.

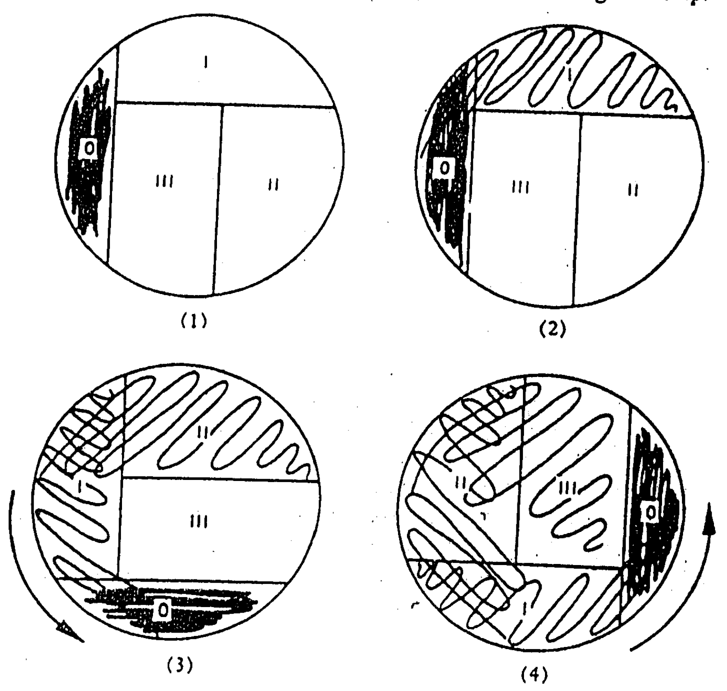


Figure 8-13: Summary of the steps used to streak for isola

MODULE 9

Cultural Characteristics of Bacteria

Discussion

Some microbes have characteristic growth patterns. Only if these growth patterns are distinctive for a single species can they aid in the identification of that species. Although some bacteria do grow in distinctive patterns, many others look very much alike . Most bacterial colonies are circular in shape with an entire margin and are raised or convex in elevation. Because many different types of bacteria have similar growth characteristics, it is possible to place too much emphasis on the study of cultural characteristics for a beginning microbiology student. Therefore, to avoid confusion, your exposure will be limited to organisms with distinctly different growth patterns. If you compare Figure 9-1 and 9-2 with figures i n other microbiology lab manuals, you will see that only the most common cultural types have been included. Figures 9-1 and 9-2 give you enough information to complete all the activities in this module, and they give an adequate introduction to the study of the cultural characteristics that you will encounter in this lab course. Study Figure 9-1 and 9-2 now, and refer to them as you perform Activities 1,2 and 3 of this module.

After studying Figures 9-1 and 9-2 , you can see that many characteristics are used attempt to categorize bacteria by their growth patterns. Three types of growth patterns are classically used:

1- An isolated cologne on the surface of a nutrient agar plate
2- A nutrient broth culture .
3- An agar slant .

Bacterial growth on these three different medium preparations has become standard for the study of cultural characteristics.

When a single species is isolated, it is a pure culture . A single isolated colony on a streak plate, a single species on a slant , or a single species in a broth are all pure cultures.

Begin now to study the morphology of an isolated colony by examining Figure 9-1. From Figure 9-1 you will learn that there are three aspects of a single colony used to study the characteristic growth features of that colony:

1- Colony shape.
2- The margin of the colony .
3- The elevation of the colony

Activity 1 tells exactly how to study and name these three colonial features.

Pigment production and different patterns of bacterial growth in broths are included when studying growing characteristics. Further explanation of pigment production by bacteria is included in Activity 4.

You will be able to complete Activity 1 during your present lab session. However, you will only be able to make the inoculations for Activities 2,3 and 4 ; they will be completed during the next lab session after they have had a chance to grow. You will find that completion of Activities 1 to 4 will allow you to do Activity 5 with ease.

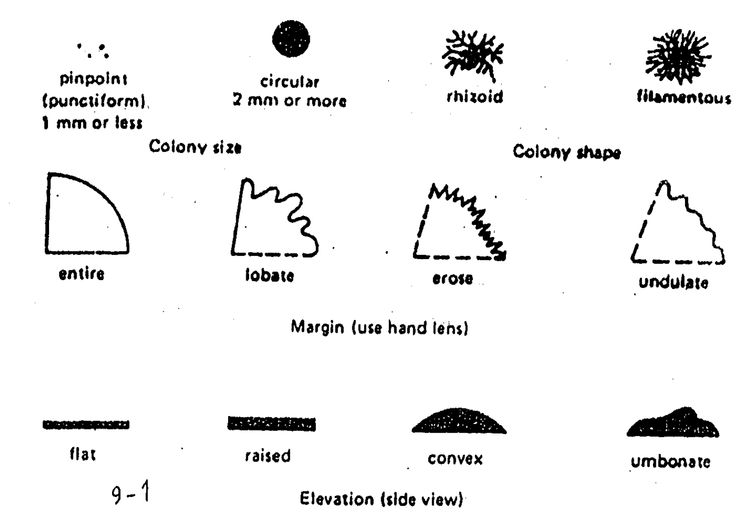


Figure 9-1: Some characterizing growth features of an isolated colony.

Activities

Activity 1 : Colony Morphology from Previously Prepared Streak Plates

Using the plates provided draw and name the following features of the colony morphology of two different colony types:

1- Shap-form of colony.
2- Margin-edge of colony. (Use a hand lens or dissecting microscope. Magnifying the edge of the colony is often necessary to determine its exact type.).
3- Elevation .

Refer to Figure 9-1 for the names of the listed colonial features for labeling your drawings. You were given two different genera of bacteria, so be sure to make a drawing and name a colony type arising from each genus.

Make drawings on the worksheet for this module. Save your best *Pseudomonas aeruginosa* plate for Activity 4.

Activity 2 : Colony Morphology from Self-streaked Plates

1- Streak one nutrient agar (NA) 1.5% plate for isolation using *Bacillus subilits*
2- Incubate at 30°C for 48 hours.
3- Streak a second NA 1.5% plate with *Proteus vulgaris.*
   - The 1.5% means that the medium contains 1.5% sodium chloride. The inclusion of NaCl causes *Proteus* to swarm over the surface in a thin film.
4- Streak a tryptic soy agar (TSA) plate for isolation using *Streptococcus pyogenes.*
5- Streak another TSA plate for isolation using *Staphylococcus aureus.*
6- Incubate these last three streak plates at 37°C for 48 hours.

After incubation, examine these plates and make drawings for colony morphology as you did in Activity 1. It may be necessary to drag your loop across the surface of your *Proteus vulgaris* plate to detect the spreading growth .

Activity 3 : Growth Patterns in Nutrient Broth

1- Use slant culture of *Escherichia coli* and the same culture of *Bacillus subtilis* you used in Activity 2 to make *aseptic* transfers of each of these organisms to a separate nutrient broth tube.

2- Next transfer *Streptococcus pyogenes* and *Staphylococcus aureus* from the slant cultures to separate tubes of nutrient broth .
   - Use your best aseptic technique since strep and staph are possible pathogens.
3- Incubate *B. subtilis* at 30°C and all other broth cultures at 37°C for 48 hours.
   - Since *B.subtilis* is not usually normal or transient flora of the human body, its optimum growth temperature is lower than body temperature (37°C).

After incubation, make drawings of all organisms grown in nutrient broth. Refer to Figure 13-2 for names and descriptions of growth types to accompany your drawings.

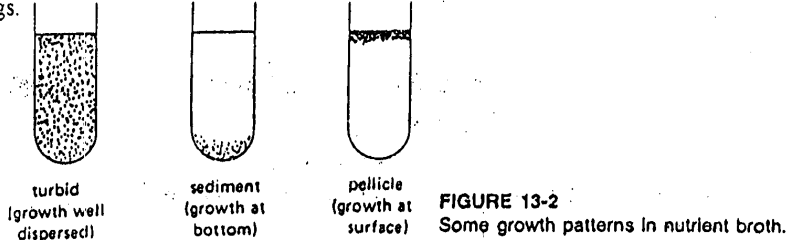


FIGURE 13-2
Some growth patterns in nutrient broth.

The pellicle of *B.subtilis* is heavy. It may break loose form the top of the tube and sink to the bottom of the broth. Look for remnants where the pellicle was attached to the sides of the tube at the surface of your broth.

Activity 4 : Production of Pigment by Bacteria

Pigment production is best studied from isolated colonies since a separated colony will allow you to see if the pigment is soluble or consolable. When we say a pigment is soluble, we mean that it is water-soluble and therefore can diffuse out of the bacterial cells making up the colony into the surrounding medium. This soluble pigment turns the medium surrounding the colony the color of that pigment. If the pigment is consolable, it remains confined inside the bacterial cells, and therefore only the colony itself is colored.

Most bacteria are not chromogenic; that is , they do not produce pigments; jemce their colonies are white or gray. The few bacteria that are chromogenic produce most colors of the spectrum, for example, green to blue, yellow to orange, red to violet, and many shades in between. A few other microbes produce a black pigment. Most pigments are n to water-soluble.

As you proceed through this course, it will become clear to you that the pigment produced by an organism can be used as another clue leading to the identification of that organism. Therefore, as you study characteristic growth features of a colony, you shouts always be observant of the various kinds of pigment production that can occur.

This activity is designed to demonstrate the difference between a soluble pigment and a nonsoluble pigment.

1- Streak a nutrient agar plate for isolation using a broth culture of *P. ıudomonas aeruginosa.*
2- Incubate at 37°C for 48 hours.
3- Use the second nutrient agar plate as a comparative control to detect pigment production after incubation.

Microscopic Observations

Organism *Micrococcus luteus* ____________

Morphology ____________

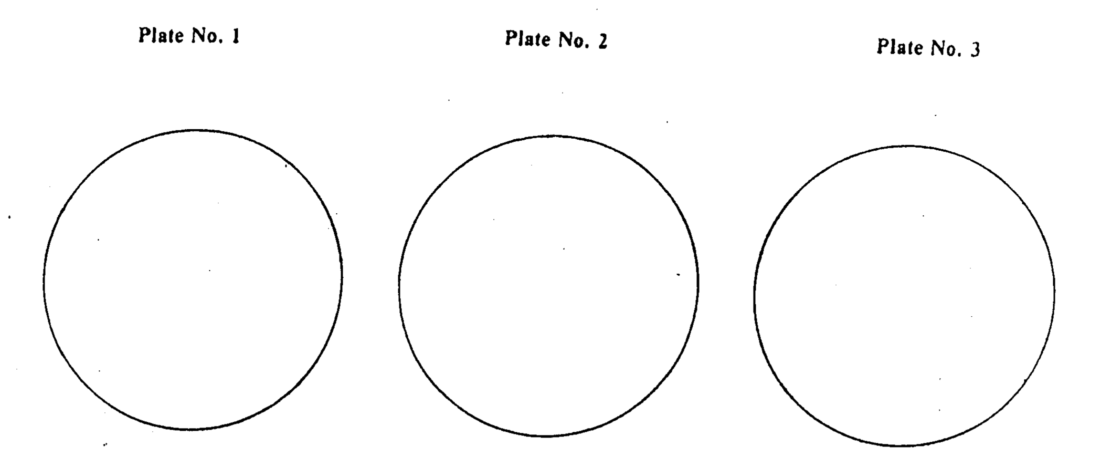
Gram Reaction ____________

Organism *Escherichia coli* ____________

Morphology ____________

Gram Reaction ____________

Pour Plate



QUESTIONS (Exercises 8 and 9)

A. **True or False Statements:** Circle the correct response.

T F 1. Contamination of cultures will arise if asepsis is not practiced in the laboratory.

T F 2 Colonies embedded in the agar of the pour plate are are smaller than surface colonies

T F 3. Streak dilution of bacterial cells means that the original inoculum has been diluted with sterile saline.

T F 4. Bacterial growth means an increase in the bacterial numbers, instead of in size of one cell.

T F 5. A magnifying glass is useful in determining the margin type of a colony.

T F 6. You should be able to pour several plates from a container holding a large amount of melted medium before you reflame the neck of the container.

T F 7. Pour plates can be used to study colony morphology.

T F 8. When you are sure the melted agar covers the bottom of a Petri plate, you can move it to the incubator before solidification.

T F 9. The water level in the holding bath must always be just above the level of the media in your tubes or bottles.

T F 10. After inoculation of the melted agar, good distribution of the bacteria will occur by diffusion if allowed to set at room temperature long enough.

T F 11. When you incubate a Petri plate, it should always be kept cover side up.

T F 12. You should flame the neck of the inoculated melted agar tube before you pour it into a sterile Petri plate.

T F 13. The original inoculum contains many more cells per milliliter than does a milliliter of the first dilution.

B. **Completion:**

14. What is the predominant shape (i.e. form) of subsurface colonies? ____________

15. Which method of separating organisms seems to achieve the best separation? ____________

16. Which method requires the greatest skill? ____________

17-18 Give 2 reasons why the nutrient agar must be cooled to 45°C before inoculating and pouring:
17 ____________
18 ____________

19 - 20. A macroscopic growth on the surface of agar is called a(n) ____________ and it represents millions of ____________ kind of cell.

Name ______________________ Date__________ Grade__________

The Streak Plate
The Pour Plate

RESULTS AND OBSERVATIONS

Streak Plate

Bacterial Species	*Micrococcus luteus*	*Escherichia coli*
Sketch of Colonies		
Size (mm)		
Form		
Elevation		
Margin		
Chromogenesis		
Light Passage*		

*Translucent - light passes through but cannot read print through colony
Transparent - can read print through colony
Opaque - no light passes through colony

Name ______________________ Date __________ Grade __________

Bacterial Motility

RESULTS AND OBSERVATIONS

Wet Mount Preparation

Organism	Motility		Description of Motility
	+	-	
Pseudomonas aeruginosa			
Bacillus subtilis			
Staphylococcus aureus			

Soft Agar Method

Organism	Motility		Extent of Growth	Amount of Growth
	+	-		
Control				
Pseudomonas aeruginosa				
Bacillus subtilis				
Staphylococcus aureus				

QUESTIONS

A. **True or False Statements:** Circle the correct response.

T F 1. Wet mounts are used more often than stained smears in the study of microorganisms.

T F 2. The numbers and location of flagella can be utilized for classification purposes.

T F 3. A motile organism migrates through the agar gel in a motility medium.

B- Complete:

4-Procayotic flagella can be classified to:
a- b- c- and d-

5- mention two types of true motility in bacteria
a-
b-

6- is a type of false motility

7- Flagella can be stained by stain

- A comparative control is an uninoculated medium that is subjected to the same physical conditions or same reagents as the inoculated medium.

In your next lab session, draw and describe the growth on this plate and your *Serratia marcescens* plate. *Pseudomonas aerugnosa* should produce a greenish water-soluble pigment. It may be helpful when comparing *P.aeruginosa* to your control plate to place them together on a sheet of white paper.

Activity 5 : Summary of Significant Cultural Characteristics

Use the descriptive names for the various growth patterns as you did in all the activities in this module. Write these descriptive names in the appropriate blanks in the table on your worksheet . This chart reinforces the fact that the same organism must be grown must be grown several different ways in order to study its cultural characteristics as described in this module.

You have now completed all the activities in this module. Be sure you have all the work that was required of you in each activity, along with the worksheet table. Not all types of cultural characteristics have been studied for all the organisms you used because of similarities amount organisms. only organisms with distinctive characteristics have been chosen for purposes of emphasis. That is why some blocks have been shaded in the table.

MODULE 10

Bacterial Viruses

Discussion

Viruses are considered noncellular because they do not have the internal structural organization and sophisticated enzyme systems of plant or animal cells. The simplest viruses are composed of nucleic acid (DNA or RNA, never both)surrounded by a protein coat. They are obligate intracellular parasites because they must use the enzymes and nucleic acids of the cells they invade in order to reproduce. This lack of self-reproducing machinery mandates that they must exist as parasites. Hence, they connote be cultivated outside of living cells. We must use bacterial cultures, susceptible plants, embryonated chicken eggs, or tissue cell cultures to grow them in the laboratory.

Viruses are very specific for the host cells they can parasitize. This allows us to divide them into three large categories:

(1) Bacterial viruses = bacteriophages
(2) Plant viruses
(3) Animal viruses

There are many different viruses in each category. Because of this specificity of host cells and virus parasites, plant viruses never infect bacteria and animal viruses never infect plants (which is why plants never get the flu!). They very narrow range of host/virus specificity depends on the complimentary surface properties of the host cell and the attachment sites of the virus.

Viruses are among the smallest ineffective particles known. Their length ranges from 25 nm to 800 nm. The poilo virus is 30 nm long, while one of the rare bacteriophages is 800 nm long. You will recall that a manometer (nm) is one-billionth of a meter or one-millionth of a millimeter. Viruses are submicroscopic and pass through a bacteriological filter with a pore size of 0.45 μm.

The bacteriophage has tow life cycles. In one cycle, called the *temperate* or *lysogenic cycle*, the phage nucleic acid is integrated with the bacterial DNA. In the *virulent* or *lytic cycle* the phage nucleic acid becomes cytoplasmic and takes over the synthetic machinery of the host cell to build 200-300 new phage particles, causing the bacterium to burst. These new phage particles can infect 200-300 more host cells, killing the host cells they parasitize.

It is the lytic cycle you will demonstrate in this module. You will seed soft agar with bacteria and dilutions of bacteriophage. The seeded soft agar mixture is poured onto the *base layer* of nutrient agar. The *seeded agar overlay* is allowed to harden and is then incubated. During incubation, bacteria grow throughout the overlay, giving it a cloudy appearance. This cloudy overlay is often called a bacterial *lawn*. Wherever a lytic phage infects a bacterial cell,, the 'continuing cycle of lysis/reinfection/lysis produces an area of clearing called a *plaque*. The number of bacteriophages in a culture can be counted by counting the plaques.This is called a *plaque assay* (see Figure10-1).

The very narrow range of host/virus specificity also makes phage typing possible. Known phage cultures are introduced into an unknown bacterial culture. If a

plaque is formed in the area of a given phage, the unknown **bacterial culture is clearly identified as the specific bacterial strain that the bacteriophage is able to parasitize.**

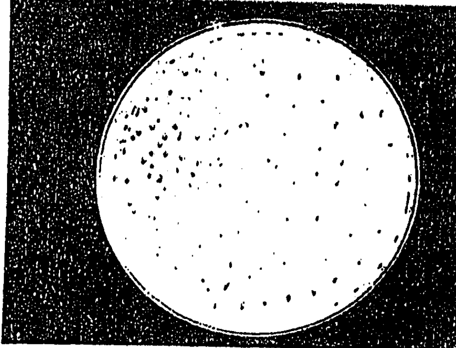


FIGURE 14-1. Plaque assay plate shows areas of clearing where phages have infected bacterial cells in an agar mixture.

ACTIVITY

Plaque Assay

Caution : Discard all pipettes into a container of disinfectant after use.

1) Place five sterile tryptose phosphate broth tubes in your tube rack.
2) Label one broth tube "control" and label the other four tubes consecutively from 10^{-4} through 10^{-7}.
3) Label five sterile tryptose agar plates the same as the tube labels.
4) Using a sterile 1 ml pipette, asepticaly transfer 0.5 ml of the 10^{-3} bacteriophage suspension provided to the broth tube labeled 10^{-4}.
5) Shake the tube well.
6) With another sterile 1 ml pipette, transfer 0.5 from the 10^{-4} tube to the tube labeled 10^{-5}.
7) Using a fresh, sterile 1 ml pipette for each transfer, repeat this diluting procedure with the remaining 2 dilution tubes (i.e. 10^{-6} and 10^{-7}).
8) With your fourth 1 ml pipette, discard 0.5 ml from your 10^{-7} tube after the ingredients are mixed.
9) Select a stock tube of *E.coli,* strain B broth culture and place in your tube rack.
10) Obtain five tubes of soft agar form the water bath and place them in your tube rack parallet to your phage dilution tubes.
    Note: You must work quickly from this point on.
11) With a sterile 5 ml pipette, aseptically transfer 0.5 ml of *E.coli*, strain Broth culture to each tube of soft agar.
12) Using a separate sterile 1 ml pipette for each , aseptically transfer 0.1 of the respective phage dilutions and the control to the corresponding tube of soft agar in the tube rack behind the dilution tubes.
13) Quickly mix the contents of each soft agar tube and aseptically pour onto the surface of the appropriately labeled tryptose agar plate (e.g., soft 10^{-4} agar tube to 10^{-4} agar plate).
14) Immediately rotate the plates gently in a 6 to 8 inch circle to ensure even distribution of the soft agar overlay.
15) Leave the plates undisturbed until the soft agar solidifies.
16) Invert the plates and incubate them at 37°C for 24 hours.

17) After incubation, examine your plates and count the number of plaques, using the Quebec colony counter and tally register. *Note:* Count only those plates having 30 to 300 plaques.
18) Record your findings (data) in the table on your work sheet. Record plates with more than 300 plaques at TNTC (too numerous to count).
19) Calculate on your worksheet the number of lytic phages per milliliter of the original bacteriophage suspension using the following formula:

$$\text{Plaque-forming units} = \frac{\text{Number of plaques formed by original viral specimen}}{\text{Dilution of original viral specimen X volume used}}$$

Name : ______________

Lab Section : ______________

MODULE 14 : BACTERIAL VIRUSES

1- Complete the following table

Dilution of virus	10^4	10^5	10^6	10^7	Control
Number of plaques					

2- Using the following formula, calculate the number of plaque-forming units in the original sample .

$$\text{Plaque- forming units} = \frac{\text{Number of plaques formed by original viral specimen}}{\text{Dilution of original viral specimen X volume used}}$$

MODULE 11

Unicellular Fungi Yeasts :

Discussion

The fungi include yeasts, molds, mushrooms, toadstools, smuts, and rusts . In nature their principal role is their power of degradation, that is , their ability to reduce organic material to inorganic molecules. In essence, they return dead organic material to the soil from which it came. Plants convert inorganic molecules, many obtained from the soil, into plant protoplasm (organic material). Animals eat the plants to make animal protoplasm. Fungi and other organisms return dead animal and plant protoplasm to the soil in the form inorganic molecules so that these in organic molecules can be used again by plants in the formation of living protoplasm.

The kingdom Fungi contains a large group of organisms that were once considered to be plants even though they are devoid of chlorophyll, roots, stems, and leaves. They range in size and complexity from one-celled yeasts to filamentous molds to complex mushrooms.

The one-celled yeasts reproduce asexually by budding. The budding daughter cell begins as a small protrusion of the mother cell as shown in Figure 11-1 . Since this is a mitotic type of division, an equal amount of genetic material is given up from the mother cell to the budding daughter cell. Therefore , the inheritable characteristics of the daughter cell are the same as those of the mother cell. After complete separation from the mother cell, he daughter cell is smaller, indicating that there is not an equal division of cytoplasm as is true in bacterial fission.

Some yeasts and molds can participate in sexual reproduction also. In the yeast (and some of the molds), this takes place by the conjugation and nuclear fusion of two fungal cells of different mating types. This is followed by meiotic divisions of the diploid nucleus, which results in ascospores. The ascospores are retained in the parent cell, which is then called an ascus (sac). The ascospores with new genetic characteristics break out of the ascus and can then germinate to produce a yeast cell with altered inheritable characteristics. Yeasts and molds that undergo this particular type of sexual reproduction in the ascus are placed in the division Ascomycota.

In this module you will be observing the more common mode of reproduction, asexual reproduction. You will need only to make a wet mount of a yeast culture to see the asexual buds arising from the mother cell. When you look at the wet mount, take on the of the size of yeast cells as compared to the bacterial cells that you have seen.

Yeasts, unlike the molds, culturally look more like bacteria when grown on artificial media. You will see this by simply observing you yeast steak plates after incubation. The physiological activities of yeast vary. For example, *Saccharomyces cerevisiae* (barker's yeast) converts simple sugars to ethyl alcohol and carbon dioxide when the condition of growth is anaerobic, while *Candida albicans* produces other nonpotable end products form the same substrate. One of the activities you will be doing in this module is designed to demonstrate the variability in the enzyme systems of these two different genera of yeasts. The yeasts that have the enzyme system to convert sugars to potable alcohol have been used by humans for centuries to make

wine and beer. The same yeast (*Saccharomyces*) is used to cause break dough to rise. We call this leavened bread.

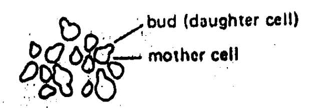


Figure 11-1 : Saccharomyces cerevisiae-Gram stain,

In 1857, Louis Pasteur proved that a certain yeast (*Saccharomyces*) produced wine by fermentation of grape sugar. He was also hired by the French government to solve the problem of "sick wine" Until that time, the bloom (organisms on the grape skins) was solely responsible for wine production. If organisms other than *Saccharomyces* predominated in the bloom the wine would have a sour taste (sick wine), since acids instead of alcohol were probably being produced. So Pasteur heated the grape mash to kill *all* the organisms; then he inoculated the cooling mash with the alcohol-producing yeast, *Saccharomyces.* This heating of wine mash was the birth of the process of pasteurization. Curing the " sick wine" made Pasteur a public hero to the wine producers and to all wine-loving Frenchmen.

Although most yeasts are beneficial to humans, a few are medically important. The two most famous of the disease-producing yeasts are *Candida albicans* and *Cryptococcus neoformans. Candida albicans* is normal inhabitant of the intestinal tract, but it becomes a medical problem in patients on prolonged antibiotic therapy. Prolonged use of antibiotics kills the indigenous bacterial flora necessary to a healthy and allows the yeast to predominate. If *Candida albicans* gets out of control in the vagina, it can be cause a most uncomfortable Volvo aginitis with much irritation and discharge. For identification, the discharge is collected by means of a vaginal swab and transported to the lab in a tube containing sterile, normal saline. Gram stains and cultures are done in order to differentiate *Candida* from other organisms that cause vaginitis. *Candida* also causes that condition known as thrush in newborn infants. Identification of *C.albicans* can often be done from the Gram stain slope. Under the microscope, *Candida albicans* looks very much like *Saccharomyces* since it is a budding yeast cell, but *Candida* differs in that bud will often elongate to form a pseudohpha, as shown in Figure 11-2. The observance of pseudohyphae is diagnostic.



Figure : 11-2 : Candida albicans-Gram stain.

Crytococcus neoformans is also a budding yeast cells and looks similar to *Saccharomyces* under the microscope. The major difference is that *Cryptococcus* has a large capsule, as shown in Figure 11-3 . If this yeast gets into the spinal fluid of a human, it causes a fatal meningitis. The diagnosis is confirmed in the laboratory, primarily by doing a negative stain on the spinal fluid obtained from the patient. A

negative stain would show encapsulated budding yeast cells if the patient has crytococcal meningitis. Figure 11-3 shows typical findings from a positive spinal fluid. (Note the presence of leukocytes-white blood cells.)

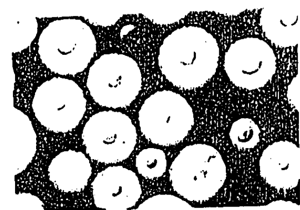


Figure 11-3 : *Cryptococcus negative stain.*

ACTIVITIES

Activity 1 : Microscopic and Cultural Characteristics of Yeast Fungi

1) Using the broth cultures of *Saccharomyces cerevisisae* and *Candida albicans*, streak each yeast for isolation on a Sabouraud dextrose agar plate.
    - Remember to shake the culture tubes gently to disperse the cells before you make your inoculations.
2) Incubate the plates at 30°C for 48 hours.
   Examine the colony morphology, and make drawings of several isolated clones of each organism on the worksheet for this module. Accompany the drawings with short written descriptions comparing the two yeasts to each other and to what you have already learned about bacterial colony morphology.
3) After incubation, make a wet mount from a colony of each yeast.

Make drawings of the microscopic appearance of each yeast. Be sure to include a few budding cells. Also include a short written description comparing the microscopic morphology of *S. cereviaiae* to *C.albicans*.

Activity 2: Physiological Differences of Yeast Fungi When Grown in Grape Juice

1) Carefully pipette 10 ml of grape juice into each of two clean screw-cap culture tubes.
2) Inoculate one tube of grape juice with a loopful of *S.cerevisiae* broth.
3) Inoculate the other tube with a similar amount of *C.albicans* broth.
    - Be sure to put the caps on tightly.
4) Incubate at 30°C for 48 hours (no longer).
5) After incubation look for bubbles of carbon dioxide gas before opening the tube.
6) Remove the cap next, look for gas again, and smell each tube for the characteristic odor of alcohol.

Activity 3 : Effect of Pasteurization on the By-products of Metabolism

Cleanliness, but not asepsis, is important in this activity, since in the past, people successfully mashed grapes with their feet.

1) Wash your hands with soap and water before handling the grapes.
2) Now rinse off the grapes with cold tap water.

- Do not wash off he bloom (grayish film on skins).

3) Cut each grape into pieces in order to get them into the culture tube.
4) Put two to three cut-up grapes into each of three clean screw-cap culture tubes.
5) Mash grapes in all three tubes with a glass stirring rod.
   - The grape pulp contains the fermentable sugars.
6) Label the three tubes 1,2, and 3.
7) Loosen the caps, and place Tubes 1 and 2 in a boiling water bath, as shown in Figure 11-4.
8) Immediately turn off the heat source, and leave the tubes in the hot water for 5 minutes to kill the mixed flora in the bloom .
9) Cool the tubes rapidly in cold water.

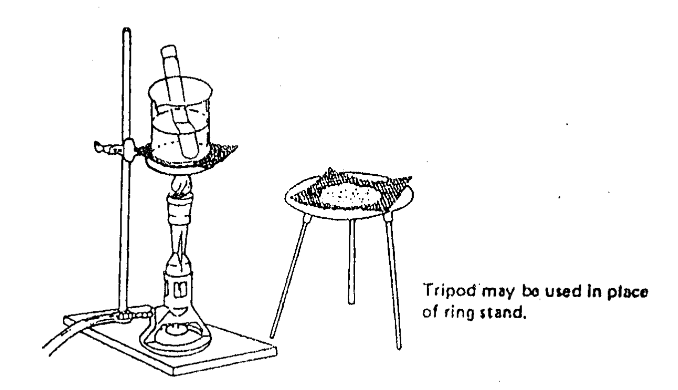


Figure 11-4: Pasteurization setup.

10) Inoculate Tube 1 with one loop of *S.cerevisiae* broth culture.
    - Do nothing more to Tube 2 after heating,
    - Tube 3 has not been heated in order to determine the effect of the still active bloom.
11) Now tighten the caps on all three tubes, and incubate them at 30°C for 48 hours.

Fill in the worksheet table for this activity, and draw conclusions about pasteurization and "sick wine"

Related Experience

Make a wet mount of the liquid from each of the three tubes from Activity 3, Did you find different organisms in Tube 3 from those in Tube 1? If you did, give a short explanation of this, demonstrating deductive reasoning.

MODULE 12

Filamentous Fungi :

Discussion

Molds are among the largest organisms studied in microbiology. Often no special staining or biochemical studies are necessary to identify the genus since their structural difference are often readily visible microscopically *if* the mold colony is not disturbed. You will be growing microcultures to look at undisturbed colones. These slide cultures can be placed directly on your microscope stage for examination of the structural differences in different mold types. The structural differences that are most often helpful in the identification of filamentous fungi are the different types of spores, spore arrangement, and hyphae.

The molds, also called filamentous fungi in order to differentiate them from yeast fungi, usually have a large, light, fluffy, colonial morphology. The light, fluffy appearance of the colony is due to its aerial hyphae and fruiting heads. So the molds differ from yeasts and most bacteria in the following ways:

1) They have different colonial morphology.
2) They are multicellular.
3) They have specialized structures that have specialized functions.
4) Their asexual reproduction is a result of the pinching off or fragmentation of one of these specialized structures.

Four divisions fungi are dealt with in this lab course:

1) Zygomycota have coencytic hypae and produce asexual spores in a sporangium.
2) Ascomycota have septate hyphae, produce unenclosed asxual spores, and produce sexual spores in a structure known as an ascus.
3) Basidiomycota are mushrooms, toadstools, smuts, and rusts.
4) Deuteromycota (*Fungi imperfecti*) are the same as Ascomycota, except no sexual spores are formed.

Following the activities in this module, you will find a much more informative taxonomic key.

In this module, you will be examining the cultureal and structural differences of a representative genus (*Rhizopus*) of the division Zygomycota and the genus *Penicillium* in the division. *Rhizopus and Penicillium* are among the many ubiquitous contamination that are considered nonpathogenic. You often see them as food spoilage organisms growing on bread, fruit, and many other foods. Therefore, as food spoilage organisms, they are merely returning protoplasm to inorganic molecules so that they can be used again by plants to build more protoplasm.

Other molds are used in the making of some cheeses. For example, the characteristic flavor and aroma of Roquefort cheese are caused by the growth of *Penicillium roqueforti* in curdled milk. The greenish-blue strands that run through the cheese are the actual mold growth itself. The mold, course, is dead if the cheese has been pasteurized. The same is true of *Penicillium camembert*, which is used to make Camembert cheese.

Another close relative of the cheese-making mold and the most famous of all molds is *Penicillium notatum*. Sir Alexander Fleming in 1929 showed that if this mold were grown in the appropriate substrate, the end product of metabolism was the wonder drug penicillin. This began the era of the discovery of antibiotics.

Although most molds are harmless or useful to humans, a few are pathogenic. Most of the pathogenic fungi are in the division *Fungi Imperfecti*. For examples of the pathogenic fungi, consult the taxonomic key.

In general, the specimens observed and cultured for laboratory identification of the systemic mycoses are sputums, lesion tissue, and lesion aspirations, whereas the superficial mycoses (dermatophytes) are usually cultured and identified from skin scrapings, nail scrapings, and broken-off hair shafts.

Before you begin the activities, inspect Figure 12-1. The will allow you to distinguish between the two classes and to identify the genus of the two nonpathogenic molds that you will be using in this module.

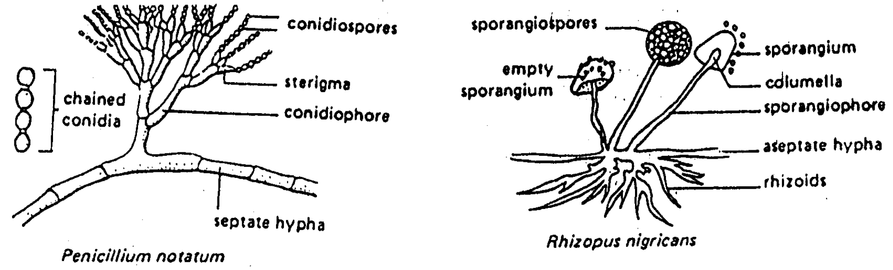


Figure 12-1 : Two genera of nonpathogenic fungi.

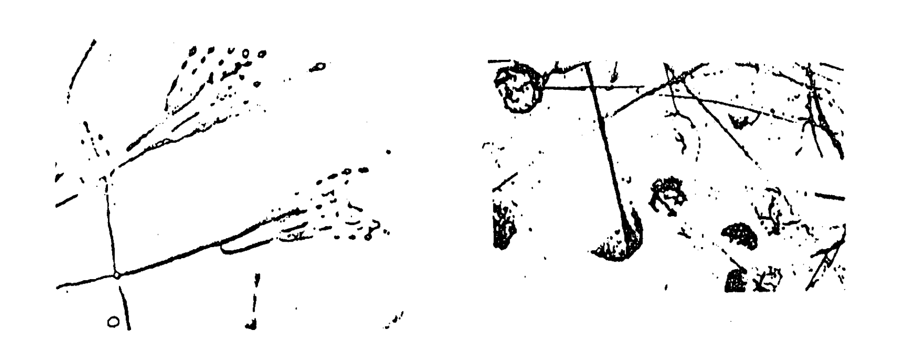


Figure 12-1 : Continued

ACTIVITIES

Activity 1: Procedure for Preparing a Microculture Using *Rhizopus nigricans*

1) Pour the Sabouraud dextrose agar into the empty, sterile petri dish, and allow it to solidify.
   - You will cut your microculture agar blocks from this plate, so use enough medium to pour a thick plate.
   - You will need only two small agar blocks from this plate, so you may wish to share it with you partner.
2) Check your sterile microculture plate. Make sure that the slide is balanced horizontally across the bent glass rod as shown in Figure 12-2.
3) Now cut a block of Sabouraud dextrose agar *no larger than 1 cm square* with your flamed inoculating loop.
4) Lift the agar block onto the slide using your inoculating loop and/or a flamed spatula.
   - Locate the agar block centrally n the microscope slide.
5) Inoculate all four upper edges of the block with *Rhizopus nigricans*.
   - Refer to Figure 12-2 for inoculating sites.
6) Now sterilize a cover slip: Dip the cover slip into a small beaker containing absolute isopropyl alcohol while holding the cover slip with a slide forceps.
   - Left most of the alcohol drain off.
7) Then quickly pass the cover slip through the flame of your gas burner once.
   - This will burn off the remaining alcohol and complete the sterilization process.
8) Place the sterile cover slip on top of the inoculated agar block.
   - Some of the hyphae will grow away from the agar block and cling to the underside of the cover slip so that they will be easily seen through your microscope.
9) Pour about 5 to 10 ml of sterile water into the bottom of the microculture plate.
   - This prevents dehydration of the agar block upon prolonged incubation.
   - Take care not to get any water on the agar block, slide, or cover slip.

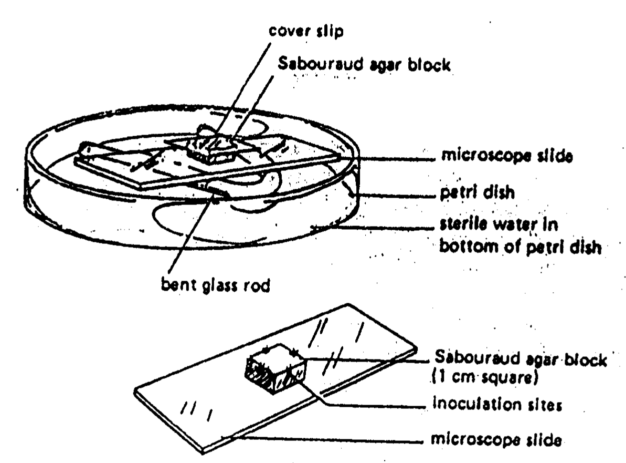


Figure 12-2 : Microculture preparation and inoculating sites.

10) Replace the petri dish lid.
11) Incubate *right side up* at room temperature for one week.

After incubation, remove the microscope slide from the petri dish, and place it on the microscope (agar block, cover slip, and all). Be sure to wipe any moisture off the bottom of the slide. Using both the low-power objective and the high-power objective, look carefilly at the hyphae, fruiting structures, spores, and rhizoids, if present. Examples of what you are likely to see are depicted in Figure 12-1 . Make labeled drawings of the specialized structures on the worksheet for this module.

Activity 2: Microculture of *Penicillium notatum*

Repeat Activity 1 exactly using *Penicillium notatum*. Make labeled drawings on your worksheet. Refer to Finger 12-1.

MODULE 13

Sterilization of Media and Equipment :

Discussion

Microbes are abundant in your environment. They are on your body, in the air, and on every surface and object that you encounter. This fact will be vividly demonstrated as you proceed through this course.

The systematic study of selected, isolated microorganisms would be impossible without some method of destroying unwanted organisms. Without effective sterilization procedures, aseptic surgery, other vital medical techniques, food preparation, and preservation methods would not be possible.

A variety of effective sterilization methods are available to us. No single method is ideal for every type of object to be sterilized, for example, methods using heat are unsatisfactory for thermometers and tend to dull cutting instruments, and chemical methods are often corrosive to metal objects. All known methods of sterilization are based on either removal or destruction of bacterial cells. In this course, your study of sterilization to methods *commonly* used in the microbiology.

Chemical sterilization in the microbiology laboratory is usually confined to the disaffection of you working area , Containers of disinfectant are supplied in the laboratory, usually beside the sinks. Make it a habit to clean the tabletops in your working area with this disiffectat *every labperiod* both *before* you begin work and *after* you finish. This habit helps to control contamination by greatly reducing indigenous microorganisms and by destroying microbes you might inadvertently scatter as you work.

Dry sterilization is used for glassware, principally petri dishes and pipettes, both of which are sterilized in cans. Dry sterilization is preferred for glassware because there is no condensation of moisture. The dry-air oven that is used for dry sterilization is simply a household oven on a larger scale. High temperatures must be maintained for long periods of time to achieve sterilization with dry heat . The routine sterilization of glassware in the dry-air oven requires a temperature of 180°C(350°F) for two hours.

Wet sterilization in the microbiology laboratory is commonly confined to sterilization by steam in the *autoclave* and/or portable steam sterilizer. Culture media, solution, and cotton (in fact, most objects sterilized in the microbiology lab except glassware or solutions decomposed by heat) are sterilized by steam in the autoclave. Hospitals. for instance , sterilize surgical packs, obstetrical packs, towels, and patient drapes in the autoclave.

The autoclave is essentially a large pressure cooker. When the autoclave is closed and the heat is turned on , it becomes a sealed container. As the water heats, pressure builds up in sealed autoclave . As the pressure increases above normal atmospheric pressure, the boiling point of the water is reaised For example, at 15 pounds per square inch (psi) above normal atmospheric pressure at sea level, water boils at 121°C(250°F) as it does at normal atmospheric pressure at sea level. Normally, all forms of life are killed by maintaining a temperature of *121°C for 15 minutes,* which is the routine sterilizing cycle for the autoclave. Because 15 psi at sea level raises the boiling point of water to 121°C ,this cycle is often expressed as "15 psi

for 15 minutes." You must remember, however, that this applies only *at sea level.* If your laboratory is not at sea level, it will be necessary to determine, by careful observation of your pressure gauge and thermometer, whether you must maintain more or less pressure to attain the necessary temperature. For example, at 2700 feet above sea level, it is necessary to maintain 20 psi to reach a temperature of 121°C. Even if the directions on a medium bottle say "sterilize at 15 psi for 15 minutes," what they really mean is to sterilize *at 121°C* for 15 minutes.

Some media, such as certain fermentation broths and skim milk deteriorate at 121°C. They must be autoclaved at lower temperatures and , therefore, at lower pressures. Table should help you to determine the pressure that you must maintain at you altitude in order to achieve the necessary temperature (121°C) for sterilization.

For example, if your directions tell you to autoclave at 7 psi for 15 minutes, you should check the table to determine the temperature corresponding to 7 psi at sea level (111.3°C). Then you must determine by careful inspection of your own autoclave pressure gauge an thermometer the pressure at you altitude that corresponds to 111.3°C.

The portable steam sterilizer can probably be most useful to you when you need to word independently , as in the identification of unknown organisms. Then you may need small amounts of several media for specific tests, and you can work independently. It is to your advantage to become thoroughly familiar with this piece of equipment, if available.

Solutions that decompose at autoclave temperatures are sterilized by *bacteriological filtration.* This method of sterilization is based on the purely mechanical process of removing all" life forms" from the solution by passing it through a filter with openings so small that bacterial cells and larger microorganisms are retained on the filter.

Several types of bacteriological filters will be available for your inspection in the lab, although you will not actually use them in this module. If you choose or your instructor so indicates, you may use a membrane filter in a future module dealing with the action of bacterial enzymes on urea. Urea decomposes at autoclave temperatures and so must be sterilized by bacteriological filtration. Inside the filter funnel is a membrane made with tiny pores of an exactly specified and controlled size. These pores are usually 0.45 um (micrometers), which is equivalent to 0.00045 mm. The average bacterial cells is larger than this and so cannot pass through the filter membrane.

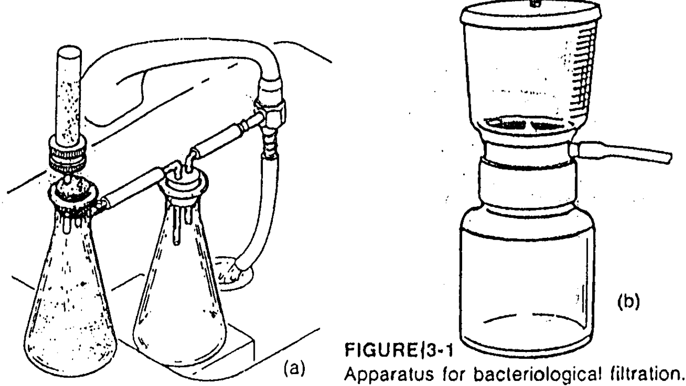


FIGURE 3-1
Apparatus for bacteriological filtration.

Table: Autoclae Steam Pressure and Corresponding Temperatures*

Steam pressure in psi at sea level	Temperature		Steam pressure in psi at sea level	Temperature	
	°C	°F		°C	°F
0	100.0	212.0			
1	101.9	215.4	16	122.0	251.6
2	103.6	218.5	17	123.0	253.4
3	105.3	221.5	18	124.1	255.4
4	106.9	224.4	19	125.0	257.0
5	108.4	227.1	20	126.0	258.8
6	109.8	229.6	21	126.9	260.4
7#	111.3	232.3	22	127.8	262.0
8	112.6	234.7	23	128.7	263.7
9	113.9	237.0	24	129.6	265.3
10#	115.2	239.4	25	130.4	266.7
11	116.4	241.5	26	131.3	268.3
12	117.6	243.7	27	132.1	269.8
13	118.8	245.8	28	132.9	271.2
14	119.9	247.8	29	133.7	272.7
15#	121.0	249.8	30	134.5	274.1

* Figures are for steam pressure only at sel level. The presence of any air in the autoclave invalidates temperature readings for any given pressure from this table.
Common sterilizing settings.

The filter funnel assembly and filter flask are wrapped and autoclaved before use. When they are set up, the solution to be sterilized is poured into the filter, and a vacuum pump is attached to the side arm of the flask. As the flask is evacuated, the solution is drawn rapidly through the filter membrane. The bacterial cells that are too large to pass trough are retained in the funnel.

All other bacteriological filters operate on the same principle regardless of the substance used for the filter. The Seitz filter employs an asbestos pad. Other filters use filter candles made of sintered glass or pad. Presterilized disposable flask and membrane filter funnel units are commercially available in various sizes.

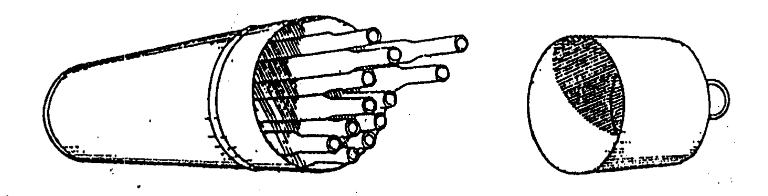


Figure 3-2 : Pipettes are sterilized in a pipette can.

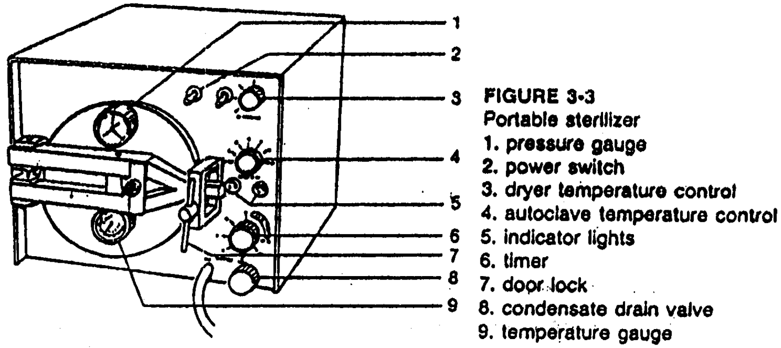


FIGURE 3-3
Portable sterilizer
1. pressure gauge
2. power switch
3. dryer temperature control
4. autoclave temperature control
5. indicator lights
6. timer
7. door lock
8. condensate drain valve
9. temperature gauge

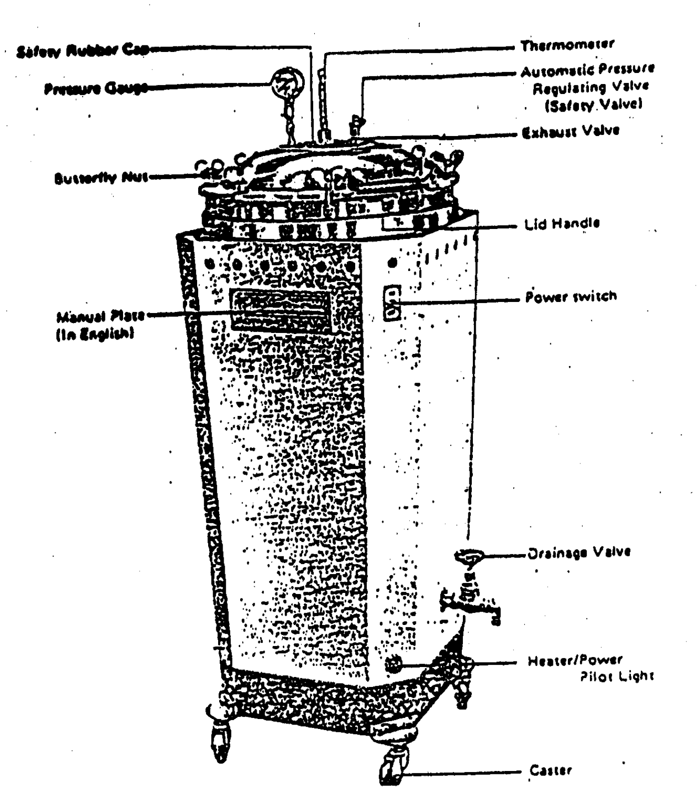
To grow bacteria to study colony morphology or to count numbers of colonies, you must grow the bacteria on or in a solid medium in a petri dish.

You will find the figures almost self-explanatory.

Accompanying the figures, you will find a checklist of the correct step-by-stepprocedure. Read the checklist while studying the figures.

Checklist

1) Assemble the materials you will need as in Figure 9-1.
2) Pick up you culture tube of melted agar (or the empty dry run tube substituting for it), and place it in your left hand
3) Remove the closure with the little finger of your right hand, as in Figure 9-2
4) Transfer the tube from your left hand to your right hand , as in Figure 9-3.
5) Flame the neck of the tube, as in Figure 9-4.
6) Simultaneously lift up the lid of the sterile petri dish and pour the melted medium into it, as in Figure 9-5.
7) If the medium does not cover the entire bottom of the plate, rotate the plate on your table in a circle 6 to 8 inches in diameter, as in Figure 9-6
8) Recap the empty culture tube, return it to your tube rack, and allow the agar plate to solidify, as in Figure 9-7



Simple Laboratory Autoclave

QUESTIONS (Exercises 2 and 3)

A. **True or False Statements:** Circle the correct response.

T F 1. An inoculum is a small amount of growth which is used to inoculate sterile media.

T F 2. The best method for sterilization of liquid media is the hot air oven.

T F 3. Aseptic technique is important when transferring microorganisms both to prevent contamination of the microorganisms you are transferring and to prevent self-infection.

T F 4. Room temperature is approximately 37°C.

T F 5. Agar is especially useful as a solidifying agent in media since nearly all bacteria use it as a nutrient.

T F 6. Pressure is the most important factor to consider when sterilizing media by autoclaving.

T F 7. Cultural characteristics play an important role in the assigning of microorganisms to particular taxonomic groups.

B. **Completion:**

8. Nutrient agar can be prepared by adding _____% agar to nutrient broth prior to sterilization.

9. A ____________________ contains only a single species of microorganism growing on or in a medium.

10. A flame-sterilized inoculating ____________________ was used to inoculate the broth and slant cultures.

11. ____________________ is another term used to describe cloudiness in a broth medium.

12.-13. Agar melts at approximately _____°C and solidifies at approximately _____°C.

14. ____________________ is a general term to describe the process which makes an object free of microorganisms.

15. An isolated population of microbial cells growing on a solid medium is called a ____________________.

16.-18. List 3 factors which might influence the cultural characteristics demonstrated by a microorganism:

16. ____________________

17. ____________________

18. ____________________

19.-20. Autoclaving takes place at _____°C at _____ psi for 15-20 minutes.

MODULE 14

Bacterial Endospores

Discussion

Bacterial endospores are small oval or spherical structures that are very resistant to high temperatures, radiation, desiccation, and chemical agents such as disinfectants. Spores are produced intracellularly by *some* bacilli, which is the reason they are called *endospores*. The ordinary bacterial cell that gives rise to the spore is called the *vegetative cell.* Endospores are smaller than the parent cells and display different qualities, most notably their great resistance to adverse conditions

The spore, however , is not formed as a response to adverse conditions. The nutritional and environmental conditions for sporulation are similar to those necessary for vegetative growth . What stimulates certain bacterial species to form spores is still unknown. Sporulation in bacteria is not a form of reproductive multiplication, as it is in some higher plants, because each cell produces only one spore, and each spore, in turn , germinates into one vegetative cell, as shown in Figure 25-1. Reproduction, then is by binary fission of the vegetative cell in spore-forming species as it is in other species of bacteria.

It appears that the spore is just a part of the life cycle of some genera of bacteria. The spore is the dormant or resting phase of the bacterial cell and *in this respect only* is analogous to the seeds of higher plants or the cysts of protozoans. The spore, however, is *not* an agent of sexual reproduction, whereas the seed is . The presence of spores in a culture is significant for identification and differentiation of bacteria since spore formation is primarily confined to the G+ rod-shaped organisms in two genera, *bacillus* and *Clostridium.*

The size and location of the spore within the vegetative cell are also significant for differentiation of organisms. For example, spores can be centrally, subterminally, or terminally located, and they can be larger or smaller in diameter than the vegetative cell. When a spore is larger in diameter than the vegetative cell, a "swelling" or enlargement and distortion of the vegetative cell results, as shown in Figure 25-2 . The sporulation character of a species are constant each time sporulation occurs and so are a useful aid in the identification of the organism.

Several spore-forming bacilli are the causative agents of disease. The anaerobic clostridia are the most famous of these. *Clostridium butulinum* causes fatal food poisoning (botulism), *C.perfringens* causes gas gangrene, and *C.tetani* causes lockjaw (tetanus). All these-forming clostridia produce powerful exotoxins that are often fatal. The most powerful of all exotoxins is produced by *C. botulinum.* The ingestion of only a minute amount of food containing botulism toxin will usually cause death. A 6 OZ. bottle of botulism toxin is sufficient to kill the entire human population on this planet.

The spores of *C.perfringens* and *C.tetani* are soil and hence on dirty objects and in food. In both diseases, the spores enter a wound with the soil or on objects with soil on them. The exotoxins of gas gangrene and tetanus are slower-acting than botulism and therefore can be neutralized by antitoxin injections. Neutralization is followed by the use of antibiotics to kill the toxin-producing bacteria and excision of the damaged tissue to remove the anaerobic environment. Indeed , early treatment is lifesaving in *C.perfringens* and *C.tetani* intoxication's.

Most species of the genus Bacillus are harmless saprophytes. *Bacillus anthracis* is the only ***aerobic*** spore-forming pathogen. The spores of this organism are also found in the soil, where they remain viable for several decades . If the spores are ingested by sheep, goats, or other animals, the disease anthrax is established. Although anthrax is primarily a disease of farm animals, it is transmissible to humans. It is an occupational hazard to farmers, veterinarians, and other handlers of infected animals because the organism can enter through a break in the skin. Workers handling animal products such as sheep's wool, goats' hair, and such can also contract the disease by inhalation of the spores. When the infection begins in the respiratory tract, it is called woolsorter's disease.

The anthrax bacillus is easily recognized microscopically. It is a large Gram-positive , spore-forming rod that forms characteristic chains. The ends of each bacillus are concave and this gives the chains a bamboo-like appearance, as shown in Figure 25-2.

Structures such as spores can often be visualized by taking advantage of certain peculiarities of the structure. For example, the bacterial endospore has very resistant spore coats. When a spore-forming organisms is stained by ordinary staining methods, the spore resists the stain and is seen through the microscope as an unstained

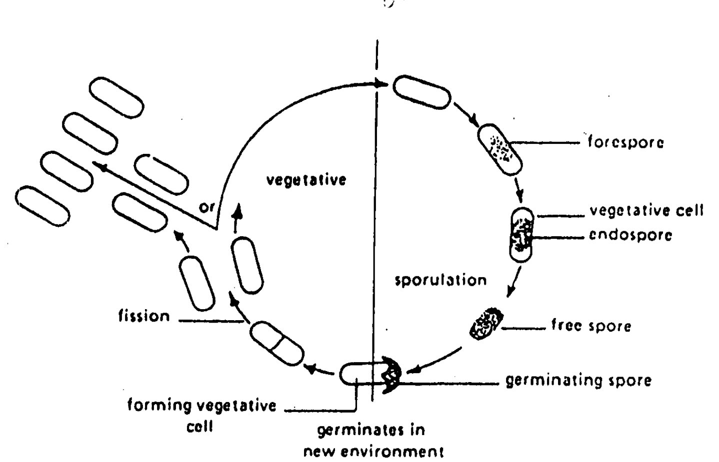
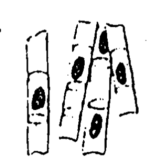
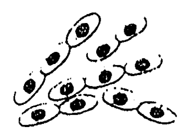
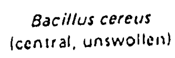


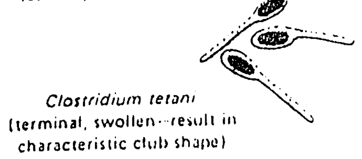
FIGURE 25-1
Life cycle of spore-forming bacteria.



Bacillus anthracis
(bamboo-like)







Clostridium perfringens
(central, swollen)

FIGURE 25-2
Size and location of some bacterial endospores within their vegetative cells.

area within the vegetative cell. However, if only a few spores are present, or if they have been released from the vegetative cells and are free in the smear, the spores can often go undetected in a simple stain or a Gram stain.

The Schaeffer-Fulton stain is a differential stain developed to visualize both the endospore and the vegetative cell. Using this method, the spore itself is stained, and free spores are easily detected. In the Schaeffer-Fulton stain, heat is used to drive the primary dye (malachite green) into the spore coats. The same characteristics of the spore that make it difficult to stain cause it to retain the dye tenaciously once it has penetrated the spore coats. The malachite green is readily rinsed out of the vegetate cell because the cell wall has been disrupted by the heating process. Therefore, the vegetative cell accepts the counter stain, safranin. When examined microscopically, the spores appear as small, green ovals or spheres within the red vegetative cells (see Figure 25-3).

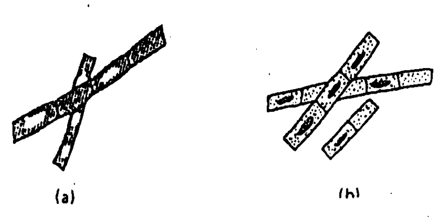


Figure 25-3 : *Bacillus subtilis*. (a) Gram stain.
(b) Schaeffer-Fulton stain.

Caution: Remember to handle all stains with the care appropriate to a potentially hazardou chemical .

Activity :

1) Prepare a smear of *Bacillus subtilis*, and heat-fix it as usual.
2) *Flood* the slide with *malachite green* stain.
3) Heat this stain-flooded slide to steaming by inverting your Bunsen burner and passing the flame over the stain periodically, as shown in Figure 25-4. When you observe steam rising from the slide, remove the burner. When the steaming stops, pass the flame over the stain again briefly. *Do not biol or allow the stain to dry. Steam* for at least *3 to 5 minutes*, replacing the malachite green if it evaporates from the slide.
4) Allow the slide to *cool* to prevent breaking it . Continue to add stain as the slide cools, since the stain is still evaporating.
5) *Drain* the slide.
6) *Rinse* with water for 30 seconds.
7) Replace the slide on you staining rack, and flood it with safranin counter stain. Allow the safranin to react for *1 minute*.
8) Drain the slide , and wash it thoroughly with water.
9) Blot the slide carefully, or allow it to air-dry.
   Examine your Schaeffer-Fulton stain with you oil-immersion objective. You should see oval or spherical green spores and red, rod-shaped vegetative . You

should see oval or spherical green spores and red, rod-shaped vegetative cells, as shown in Figure 25-3b. On your worksheet, draw several representative cells, and label the spores and vegetative cells.

Practice the Schaeffer-Fulton stain procedure as often as you feel necessary. Then take the post test.

Formulae for Reagents

1) MALACHITE GREEN (5% aqueous):

Dissolve 5.0 g of malachite green in 100.0 ml of distilled water.

2) SAFRANIN COUNTER STAIN (0.5% AQUEOUS)

Dissolve 0.5 gm of safranin in 100.0 ml of distilled water.

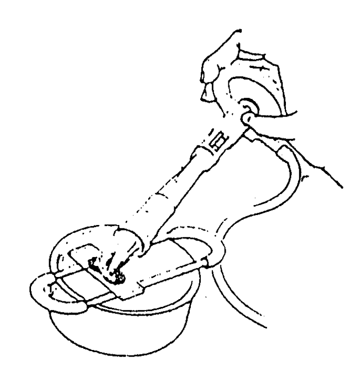


Figure 25-4 : Heating process of the Schaeffer-Fulton stain procedure.

MODULE 15

Media In Common Use

The medium on which bacteria are grown will vary in its composition according to the requirements of the particular apices. Some bacteria will grow well on a very simple medium containing only inorganic salts plus an organic carbon source, such as sugar. Others- particularly the pathogenic species require a every complex medium to which blood or other complex materials are added. Almost all routine media can be purchased commercially as dry powders. Thus to prepare a medium, one need only weigh out the desired amount of powder, add water, dispense, and sterilize before use.

I. Ordinary or Basic Media :

a) Fluid Media :

1- Nutrient broth :

One of the most common media used for the routine cultivation of bacteria which is prepared by boiling ground meat with water and filtering off the solid material to yield a clear liquid called an infusion. Peptone, which is partially degraded protein, and frequently, 0.5 percent sodium chloride (common table salt), are added to the liquid to provide carbon, nitrogen, and certain inorganic substances for bacterial growth. After the pH is adjusted so that the material is neither too acid nor too alkaline, the medium is ready for sterilization and, subsequently, for use as a bacterial medium. Usually the medium is dispensed in screw cap tubes or test tubes that are plugged with nonabsorbent cotton before they are sterilized. The more common bacteria grow in this medium to give a uniform cloudiness (turgidity). This turgidity is evidence of growth.

2- Peptone water :

This is a simpler medium. 1% peptone and 0.5% NaCl in water, dissolved by heat and filtered. The filtrate which is a clear colour less fluid is sterilized in the autoclave. This medium is mainly used to test for insole production from peptone and is also used as a basis of sugar media.

b) Solid Media :

1- Nutrient agar medium:

To grow bacteria on a solid surface rather than suspended in a liquid broth, 1.5 to 2 percent agar is added to the broth to make solid. Once the agar has been melted in the medium it can be dispensed in tubes which then are plugged with cotton and sterilized. After sterilization, and while the mixture is still melted, the tubes sometimes are slanted so that after solidification there will be a large surface area to use for bacterial growth, this type is usually referred to as a slant, in contrast to a somewhat fuller tube which has bee allowed to solidify in an upright position, called a deep.

2- Gelatin medium :

This medium contains 10-15% of gelatin dissolved in broth. This medium is sterilized in an open autoclave for 20 or 30 minuets three successive days. It is solid below 24^{o}C . The medium is acted on and liquefied by prototypic organisms. It is used for detection of proteolytic activity of microorganisms.

II- Enriched Media :

Certain organisms, e.g. gonococcus, pneumococcus etc., will not grow on the ordinary media described, they require more complex organic body fluids for their growth. Media containing such substances are called enriched media.

The following are the most commonly used enriched media:

1- **Blood Agar :** Ordinary agar melted at 100^{o}C and allowed to cool to 55^{o}C , while it is still liquid, 5-10% startled efibrinated blood of sheep, ox or horse, are added and mixed well with the melted agar. The medium is then poured in plates or slopes and allowed to cood and solidify. It is an opaque and medium and is very useful not only as an enriched medium but also as an enriched medium but also as indicator medium , differentiating organisms according to their action on blood ; some causing complete haemolysis as *Staph. aureus* and *Strept. haemolyticus*, others causing greenish coloration as *Strept. viridans* and pneumococcus and others producing no change.

2- **Chocolate agar :** or heated blood agar prepared as blood agar but after adding the blood to the melted agar at 55^{o}C. the temp. is raised to 100^{o}C for 2 minutes then poured in plates or slopes. The medium is used particularly for the culture of the haemophilus group of organisms.

3- **Serum agar** : Prepared as blood agar using sterile serum instead of blood .

4- **Serum broth :** 5% sterile serum added to broth :

5- **Lofflers serum :** Contains horse or ox serum and glucose broth, in slopes and put in the hot air oven at 75^{o}C for hours, on 2 successive days. The medium solidifies into an opaque whitish medium used for the culture of *Coryn. diphtheria*

6- **The Lowenstein Jensen Medium :** Is a special agg medium containing glycerine potato starch, asparagine and malachite green. The latter renders the medium selective for T.B. and inhibits other organisms. It is an opaque greenish medium.

III. Carbohydrate Media :

Various sugars (carbohydrates) may be added to a nutrient broth , base. This type of medium is used to determine if the bacterial species being identified is able to use a particular sugar for growth. Commonly employed in media of this type are such sugars as glucose , mannose, galactose, sucrose, maltose, and lactose. In addition, some sugar alcohols such as mannitol, glycerol, and dulcitol are used.

When a sugar is used by a bacterium usually both acid and gas are produced. Since were cannot see either the acid being produced or the sugar disappearing ,a dye

(called an indicator) which changes color in the presence of acid is added to the medium. Thus, to determine if the sugar in question was used, one need only observe the color of the medium after 24 to 48 hours of growth . Similarly, to determine if gas is produced, we can trap the gs so that it can be seen. This is accomplished by placing a small Durham's tube (upside down) in the carbohydrate medium. If gas is produced, it will be trapped inside the tube (displacing the liquid medium), and we shall be able to see gas bubbles.

IV. Selective and Differential Media :

Dyes frequently are added to a medium to inhibit the growth of certain bacteria while not interfering with the growth of others. This type of medium is called a *selective medium*, since it will select certain organisms. The addition or bile salts to a medium can make it selective for the pathogenic enteric organisms in the genera *Salmonella* and *Shigella*, Such a medium will partially inhibit the growth of *Excherichia coli* but will allow the pathogenic organisms to grow.

As noted previously, an acid indicator may be added to a solid medium so that a colony o bacteria that forms acid can be differentiated from one that does not. This type of medium is called a differential medium. Both selective and differential media are particularly useful in the identification and the isolation of enteric pathogens. For example, *E scherichia coli*, a normal inhabitant of man's intestinal tract, ferments lactose to form acids. On the other hand , many disease-producing organisms of the intestinal tract (such as the typhoid organism) do not ferment lactose to form acid. Thus, in the primary culture, colonies of the typhoid organism can be differentiated from *E.coli* on a differential medium such as MacCConkeys which contains an indicator that will be changed in color by the lactose users.

V. Enrichment Cultures :

Bacteria present in very small numbers in some natural environment are frequently isolated with difficulty from the mixed population. If a suitable substrate and other conditions are provided that favor the growth of the orgasms but are unsuitable for others, they can become dominant . Liquid media are used for enrichment cultures. The medium provides nutrients and environmental conditions that favor the growth of the particular organism but are not suitable for the growth of other types. After repeated transfers in enrichment media, differential plating methods can be used for pure culture isolation. For example, selenite broth is used to enrich Salmonella.

VI. Characteristic Media :

Characteristic media are utilized to test organisms for a particular metabolic activity, products, or requirements. The type f reaction observed on these types of media is helpful in identification.

1. Triple Sugar Iron (TSI) :

Triple sugar iron contains lactose. , sucrose, and glucose plus ferrous ammonium sulfate and sodium thisulfate. TSI is generally used for the identification of enteric organisms by their ability to attack glucose, lactose, or sucrose and to liberate sulfides from ammonium sulfate or sodium thiosulfate.

2. Lysine Iron Agar (LIA)

Lysine iron agar is utilized to differentiate organisms which can either deaminate or decarboxylize the amino acid lysine. LIA contains lysine, which permits enzyme detection, and sodium thiosulfate, which liberates sulfides.

3. Sulfide, Indole, motility (SIM):

Sulfide, indole, motility medium can perform three different tests. One can observe the production of sulfides, formation of indole, a metabolic product from tryptophan utilization, and, finally, motility. This medium is generally used for the differentiation of enteric organisms.

VII. Chemically Defined Media:

A chemically defined medium is one in which the exact chemical composition is known. Gastrock's medium is an example of chemically defined media.

VIII. Media for Cultivation of Yeasts and Molds:

Fluid Sabouraud's and Sabouraud's agar are recommended for the cultivation and maintenance of yeasts and molds. The composition of Sabouraud's agar is as follows:

Glucose	40 gm.
Peptone	10 gm.
Agar	15 gm.

Distilled water to 1 litter.
Final pH 5.6

The low pH an the high concentration of sugar (4%) are inhibitory to bacteria. Both tube and plating medium can be prepared.

MODULE 16

Biochemical Testing of Microorganisms

BIOCHEMICAL TESTS USED TO DIFFERENTIATE BACTERIA

Test	Main Use	Biochemical Reaction	Positive Test
Arylsulphatase	To differentiate *Mycobacterium* species	The enzyme arylsulphatase releases phenolphthalein from its sulphate and this is detected by adding an alkali	Pink-red medium
Bile solubility	To distinguish *S. pneumoniae* from viridans streptococci	Bile salts dissolve pneumococci but not other streptococci	Clearing of turbidity
Catalase	To differentiate staphylococci from streptococci	The enzyme catalase breaks down hydrogen peroxide to oxygen and water	Release of oxygen bubbles
Citrate	To differentiate enterobacteria from other bacteria	Organism uses citrate as its only source of carbon, producing an alkaline reaction with a colour change of indicator	Blue and turbid medium
Coagulase	To identify *S. aureus*	The enzyme coagulase clots plasma	Tube: Fibrin clot Slide: Clumping of bacterial cells
DNAse	To identify *S. aureus*	The enzyme deoxyribonuclease hydrolyzes DNA	Clearing around colonies
Hydrogen sulphide (H_2S)	To differentiate enterobacteria, *Bacteroides* species, and *Brucella* species	Sulphur-containing amino-acids are decomposed with the release of H_2S which is detected by an iron salt	Blackening in medium
Indole	To differentiate Gram negative rods, especially *E. coli*	Tryptophan is broken down with the release of indole which reacts with dimethylaminobenzaldehyde	Reddening of strip or medium
Litmus milk decolorization	To identify enterococci and some *Clostridia*	Litmus milk is reduced with a decolorization of the litmus	Medium becomes white or cream coloured
Nitrate reduction	To differentiate *Mycobacterium* species and Gram negative bacteria	The enzyme nitrate reductase reduces nitrate to nitrite which diazotizes sulphanilic acid. This reacts with α-naphthylamine	Reddening of medium
Oxidase test	To help identify *Vibrio*, *Neisseria*, *Pasteurella*, and *Pseudomonas* species	Oxidase enzymes oxidize phenylenediamine	Deep purple colour on reagent paper

Oxidation-fermentation	To help identify *P. aeruginosa* and differentiate other bacteria	Aerobic (oxidative) and anaerobic (fermentative) utilization of carbohydrate	Oxidative: Yellow in open tube, green in closed tube. Fermentative: Yellow in both tubes
Phenylalanine deaminase	To differentiate *Proteus* and *Providencia* from other enterobacteria, and exclude *Y. enterocolitica*	Phenylalanine is broken down with the production of phenylpyruvic acid. The acid is detected by iron III chloride	Slope becomes green
Tween hydrolysis	To identify slow-growing *Mycobacterium* species	The enzyme lipase hydrolyzes Tween 80, producing oleic acid which changes the colour of the indicator	Pink-red substrate
Urease	To help identify *Proteus*, *Morganella*, and *Y. enterocolitica*	The enzyme urease hydrolyzes urea, producing ammonia which changes the colour of the indicator	Red-pink medium
Voges-Proskauer (V-P test)	To differentiate enterobacteria	Organism ferments glucose with acetoin production. Acetoin is oxidized to diacetyl which reacts with creatine	Pink colour developing slowly in medium
Methyl red	To differentiate enterobacteria	Organism ferments glucose, producing sufficient acidity in a buffered medium to give a colour change of indicator	Bright red medium

QUESTIONS

A. **True or False Statements**: Circle the correct response.

T F 1. The Durham tube allows you to determine the kind of gas produced during fermentation.

T F 2. The formation of alpha-keto acids results in an acid condition.

T F 3. Gas production must be preceded by acid production.

T F 4. The endoenzymes of fermentation are secreted into the environment.

T F 5. If phenol red turns a magenta color, it means ammonia has been produced.

B. **Completion:**

6.-7. The small inverted tube found within a larger tube is called a ____________________ tube and is used to trap ____________________.

8. When phenol red changes to yellow, this indicates that ____________________ has been produced.

9.-10. The two pH indicators most often used in a microbiology laboratory are ____________________ and ____________________.

11.-12. The process of fermentation involves 2 groups of enzymes known as ____________________ and ____________________.

13.-16. List the 4 ingredients of a fermentation tube.

13. ____________________ 15. ____________________

14. ____________________ 16. ____________________

17.-20. List 4 fermentable carbohydrates other than those used in this exercise.

17. ____________________ 19. ____________________

18. ____________________ 20. ____________________

21. ____________________ is the term that refers to the anaerobic breakdown of carbohydrates.

22.-25. What is the pH range of the indicator used in this exercise? ____________________
What colors denote a neutral, acid or alkaline reaction?

neutral ____________________

acid ____________________

alkaline ____________________

26. If a carbohydrate broth does not change color after it has been inoculated and incubated, how can you tell whether the unchanged color is due to failure of the organism to grow or failure to ferment the carbohydrate?

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Name ______________________ Date__________ Grade__________

IMViC Reactions

RESULTS AND OBSERVATIONS

Organism	**IMViC Reactions**			
	I	M	Vi	C
Control				
Escherichia coli				
Enterobacter aerogenes				

+ = positive test
− = negative test

QUESTIONS

A. True or False Statements: Circle the correct response.

T F 1. Kovac's reagent must be added to MR-VP medium to detect the results of the Voges-Proskauer test.

T F 2. Indole is a breakdown product of tryptophane.

T F 3. The *Enterobacter - Klebsiella* groups are mixed acid fermenters.

T F 4. The fermentation of lactose is the basis for the methyl red test.

T F 5. 2,3 butanediol producers can be methyl red positive if measured too early.

B. Completion:

6.-7. Name 2 types of media that can be used to detect citrate utilization.

6. ____________________
7. ____________________

8. ______________________ is the medium used to test for indole production.

9. ______________________ is the chemical reagent used in the indole test.

10. The pH must be below ______________________ for a positive methyl red test to occur.

11.-12. Name the 2 major products produced by Voges-Proskauer positive organisms.

11. ______________________

12. ______________________

13.-14. ______________________ and ______________________ are the reagents used in the Voges-Proskauer test.

15. Members of the Enterobacteriaceae are commonly referred to as the enteric bacilli or ______________________.

16. Those enterics that do not fall into the 2 distinctive categories formed by *Escherichia coli* and *Enterobacter aerogenes* are collectively referred to as ______________________.

17.-24. Indicate the color of positive and negative tests for each of the following:

Reaction	Positive Test	Negative Test
Indole	17. ______________	21. ______________
Methyl red	18. ______________	22. ______________
Voges-Proskauer	19. ______________	23. ______________
Citrate	20. ______________	24. ______________

Name ______________________ Date__________ Grade__________

Catalase Production

RESULTS AND OBSERVATIONS

Organism	Catalase Production	
	+	-
Control		
Streptococcus faecalis		
Staphylococcus aureus		

QUESTIONS

A. **Completion:**

1.-2. ____________ is a respiratory enzyme found in most aerobic organisms. It is tested for by the addition of the chemical reagent ____________.

3.-6. The catalase test is especially valuable in distinguishing between the Gram-positive cocci: ____________ (genus) (catalase +) and ____________ (genus) (catalase -), and in distinguishing between the Gram-positive long rods: ____________ ______ (catalase +) and ____________ (catalase -).

7. ____________ organisms do not produce catalase and are poisoned by the accumulation of hydrogen peroxide.

Name ______________________________ Date__________ Grade__________

Oxidase Production

RESULTS AND OBSERVATIONS

Organism	Oxidase Production	
	+	-
Control		
Pseudomonas aeruginosa		
Escherichia coli		

QUESTIONS

A. **Completion:**

1. An obligate anaerobe would be expected to give a ____________________ test for oxidase.
2. A ____________________ color appears in a positive oxidase test.
3. The chemical ____________________ is added to test for the production of oxidase.
4. A positive oxidase test depends upon the presence or absence of ____________________ in the cell.
5. Oxidase production has no value in classification. True_____ or False_____

B. **Completion:**

6.-7. List the reagents used to test for nitrite.

6. ____________________

7. ____________________

8.-10. Give the reaction color for the following when sulfanilic acid and dimethyl-α-naphthylamine reagents are added.

Reaction	**Reaction Color**
$NO_3 \rightarrow NO_2$	8. ____________________
NO_3	9. ____________________
$NO_3 \rightarrow NH_3$	10. ____________________

11. ____________________ is one enzyme that reduces nitrate to nitrite.

12. ____________________ is the enzyme that reduces nitrite.

13. Is the reduction of nitrate to nitrite favored by aerobic or anaerobic conditions? ____________________

14. It is preferable to perform this test by periodic testing of some of the culture over a period of several days. Why? ____

15.-17. Explain each of the following:

15. Nitrite test negative, no nitrate present ____________________________________

16. Nitrate test negative, nitrite present ____________________________________

17. Nitrite test negative, nitrate present ____________________________________